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Letter to the Editor

Note: The editor assumes no responsibility for the statements and opinions expressed by the contributors. This issue is delayed due to unavoidable circumstances.

Indian Journal of Malariology
Vol. 35, December 1998, pp. 171-177.

Stage-specific Effects of Antimalarials on an Indian Isolate of *Plasmodium falciparum*

D. GHOSH and S.K. SUBBARAO[a]

In this study, effects of timed sequential exposure to various concentrations of chloroquine and its two metabolites (SA100387/050 B, SA 100487/053 B), quinine, hydroquinine and quinidine were monitored by morphological analyses, using synchronous cultures of *Plasmodium falciparum*. The trophozoites and schizonts were sensitive to chloroquine and its metabolites. However, quinine and two of its derivatives were lethal without particularly being stage-specific.

Keywords: Chloroquine, Hydroquinine, Metabolite, *Plasmodium falciparum*, Quinidine, Quinine

INTRODUCTION

Although antimalarial drugs, such as chloroquine and quinine, have been intensively studied, the exact mechanism(s) of action of these compounds are not well defined in *Plasmodium falciparum*. With the increase in drug resistance, the use of existing drugs need to be optimized; new drugs and drug combination be devised and their mechanism of action be explored.

In vitro studies have made it possible to explain the mechanism of action by eliminating the complicated host factors. Many theories have been proposed[1,2] regarding, chloroquine and quinine, the derivatives of quinoline, all of which conform to the hypothesis that the drugs attack the mature stages (schizonts and trophozoites) of the malarial parasites. However, Zhang *et al.*[3] demonstrated that chloroquine inhibits the parasite at the ring stage only. This was in contradiction to the traditional concept of chloroquine being a blood schizontocide.

In this study, we report our findings on the effects of chloroquine, its two metabolites,

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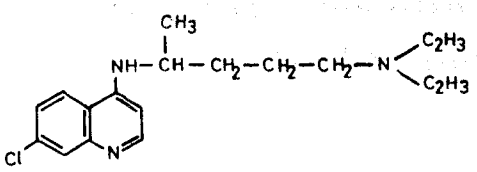
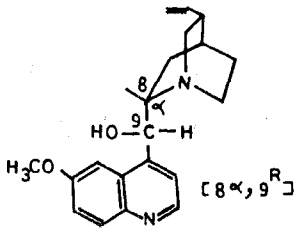
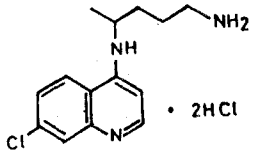


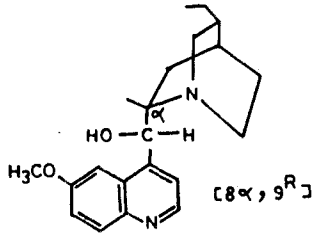
Chloroquine
7-chloro-4-(4'-diethylamino-1'
-methylbutylamino)quinoline



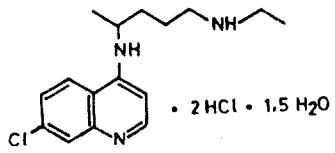
Quinine
(Code: Ph4008)
6-methoxy-α-(5-vinyl-2-quinuclidinyl)
-4-quinoline methanol



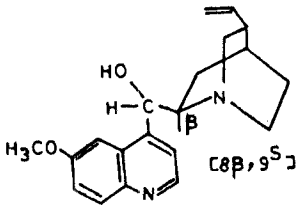
Metabolite of chloroquine
(Code: SA100387/050B)
7-chloro-4-(4-amino-1-methyl
-butylamino)-quinoline.2HCl



Derivative of quinine
(Code: Ph4017)
Hydroquinine (base)



Metabolite of chloroquine
(Code: SA100487/053B)
7-chloro-4-(4-amino-1-methyl
-butylamino)-quinoline.2HCl.1.5H_2O



Derivative of quinine
(Code: Ph4012)
Quinidine (base)

Fig. 1: Structures of antimalarials used in the study (Derivatives were provided by Dr. Peter Buchs)

quinine and two of its derivatives on a cultured Indian isolate of *Plasmodium falciparum*.

MATERIALS AND METHODS

The isolate, named as PfMD-C, was obtained from Malaria Research Centre, Delhi and thereafter established *in vitro* using the method of Trager and Jensen[4]. The details of all the drugs used in the present study are given in Fig. 1.

Synchronization of culture

The culture was synchronized by sorbitol lysis ($\geq$ 90% rings)[5], followed by a second synchronization, 40 h after the first one.

Stage-specific effects of the drugs

Stock solutions of drugs (1×10^{-3} M) were prepared. Chloroquine and its metabolites were water soluble, whereas quinine and its derivatives were prepared in 0.1 N H_2SO_4. Synchronous infected erythrocytes, with a parasitaemia of 1-1.5%, were divided into four test groups and cultured in 24-well microtitre plates.

Group 1: As control group where parasites were cultured in drug-free medium.

Group 2: Parasites were cultured at ring stage, i.e. at 0 h, in the medium containing various concentrations of the drugs used (0.003×10^{-6} to 0.3×10^{-6} M) after synchronization.

Group 3: Parasites were exposed to drugs at trophozoite stage, i.e. parasites were grown in drug-free medium from 0-24 h and cultured thereafter in medium containing various concentrations of the drugs.

Group 4: Parasites were exposed to drugs at schizont stage, i.e. parasites were cultured in drug-free medium from 0-37 h and then cultured in a medium containing various concentrations of the drugs.

All experiments were carried out in duplicate. Thin blood films of the cultures were made at 0, 24, 37 and 48 h respectively. The slides were fixed with methanol and stained with Giemsa. The ring, trophozoite and schizont stage parasites per 5000 erythrocytes were determined by microscopic examination. A linear regression analysis was done and the IC_{50} and IC_{90} values (concentration indicating 50 and 90 per cent inhibition) were determined.

RESULTS AND DISCUSSION

It is necessary to know the residence time of each of the asexual stages of the parasite (i.e. rings, trophozoites and schizonts) during 48 h schizogonic cycle, so as to ensure the exposure of parasites to the drug during the residence time of the particular stage.

Group 1: Almost all the parasites were in ring stage at 0 h, trophozoites accounted for more than 95% of the parasites at 24 h, the highest distribution of schizonts was at 37 h. New rings were maximum at 48 h. The

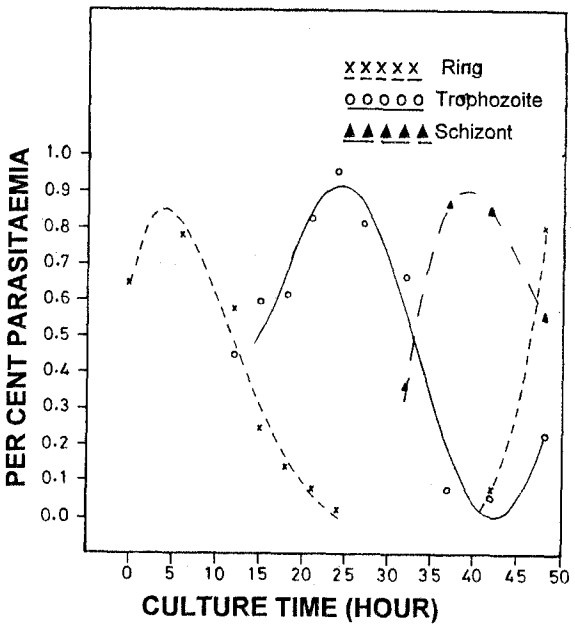


Fig. 2: Synchronization of erythrocytic stages of the parasite isolate in culture

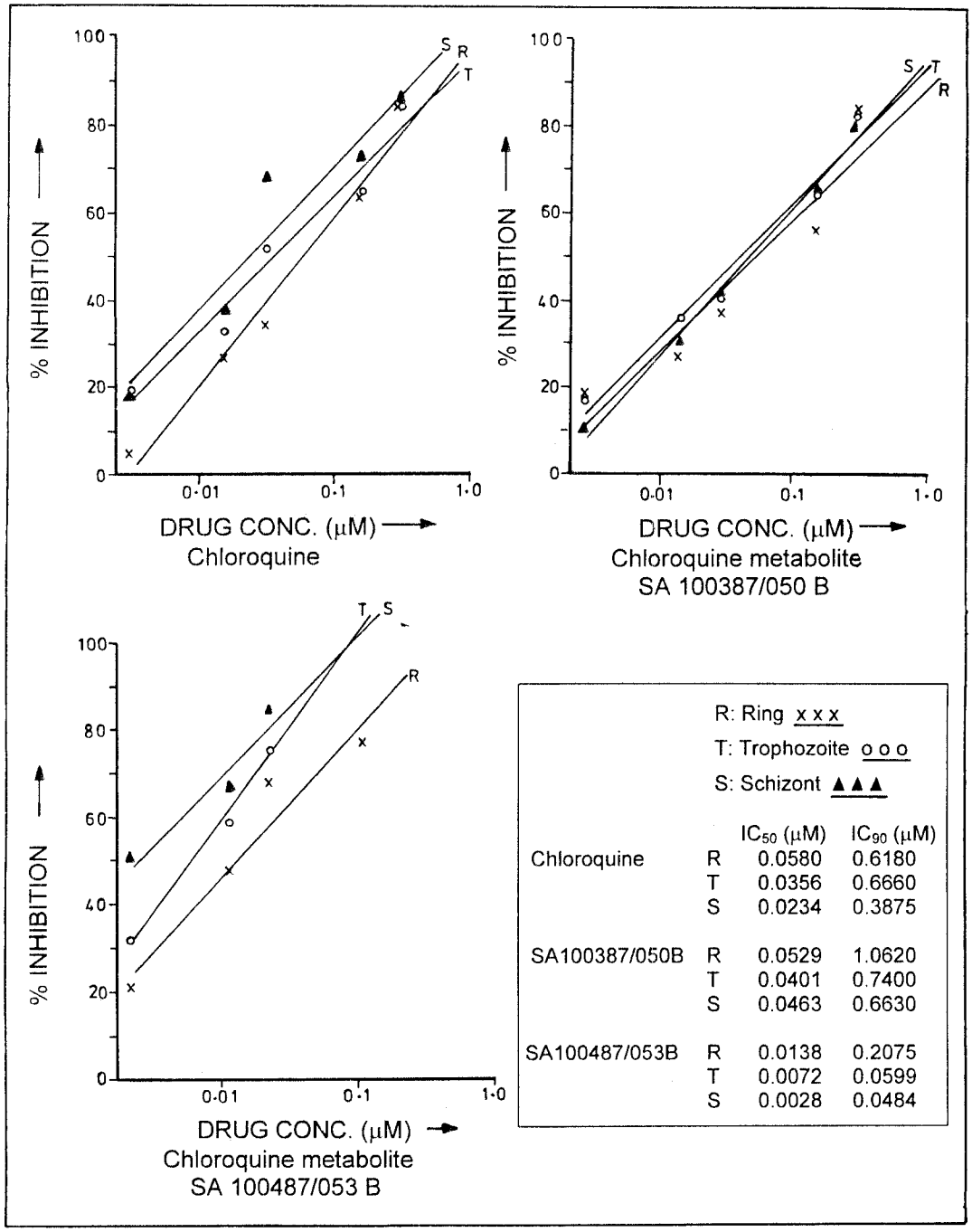
distribution pattern over the cycle is presented in Fig. 2.

Group 2: The growth of the parasites from ring to trophozoites remained unaffected in case of chloroquine and its metabolite SA-100387/050B. However, the other metabolite of chloroquine SA100487/053B, quinine, hydroquinine and quinidine arrested the growth of the parasites from ring to trophozoite stage. Most parasites were killed at ring stage within 24 h. The morphology of the rings appeared to be pycnotic, large and vacuolated.

Groups 3 and 4: As compared to the control, in these groups, the growth of the parasites were arrested both at trophozoite and schizont stages. The parasites were resistant to both chloroquine and its metabolite SA100387/050B. Trophozoite and schizont stages were more sensitive to chloroquine metabolite SA100487/053B, quinine, hydroquinine and quinidine.

The concentration-effect curves for the various asexual stages are presented in Figs. 3 and 4. This demonstrates that the trophozoite and schizonts are in general more sensitive to chloroquine and its metabolites than the ring stages, except SA100387/050B, whereas all the stages appeared to be equally sensitive to quinine and its derivatives. It was observed that a shift in the drug sensitivity appeared to occur in the late ring stages, when they become morphologically distinguishable as trophozoites. This change in drug sensitivity could be due to changes in the rate of uptake of the drug by the parasite, changes in the presence of intracellular factors or components necessary to accumulate the drug inside the parasitized erythrocyte, changes in the number of chloroquine or quinine receptor sites or changes in the activity of a metabolic process that is specifically inhibited by the drug.

The effective action of chloroquine on trophozoite and schizont stages led to the belief that possibly the drug interferes with polyamine synthesis, as the mature stages of the parasite are rich in it[6,7]. Based on the prior data and the results of the present work, it may be assumed that chloroquine and its metabolites act on the mature stages of the



		IC_{50} (μM)	IC_{90} (μM)
Chloroquine	R	0.0580	0.6180
	T	0.0356	0.6660
	S	0.0234	0.3875
SA100387/050B	R	0.0529	1.0620
	T	0.0401	0.7400
	S	0.0463	0.6630
SA100487/053B	R	0.0138	0.2075
	T	0.0072	0.0599
	S	0.0028	0.0484

Fig. 3: Stage-specific effects of the antimalarials (chloroquine, SA100387/050B, SA100487/053B) in synchronous cultures of the parasite isolates

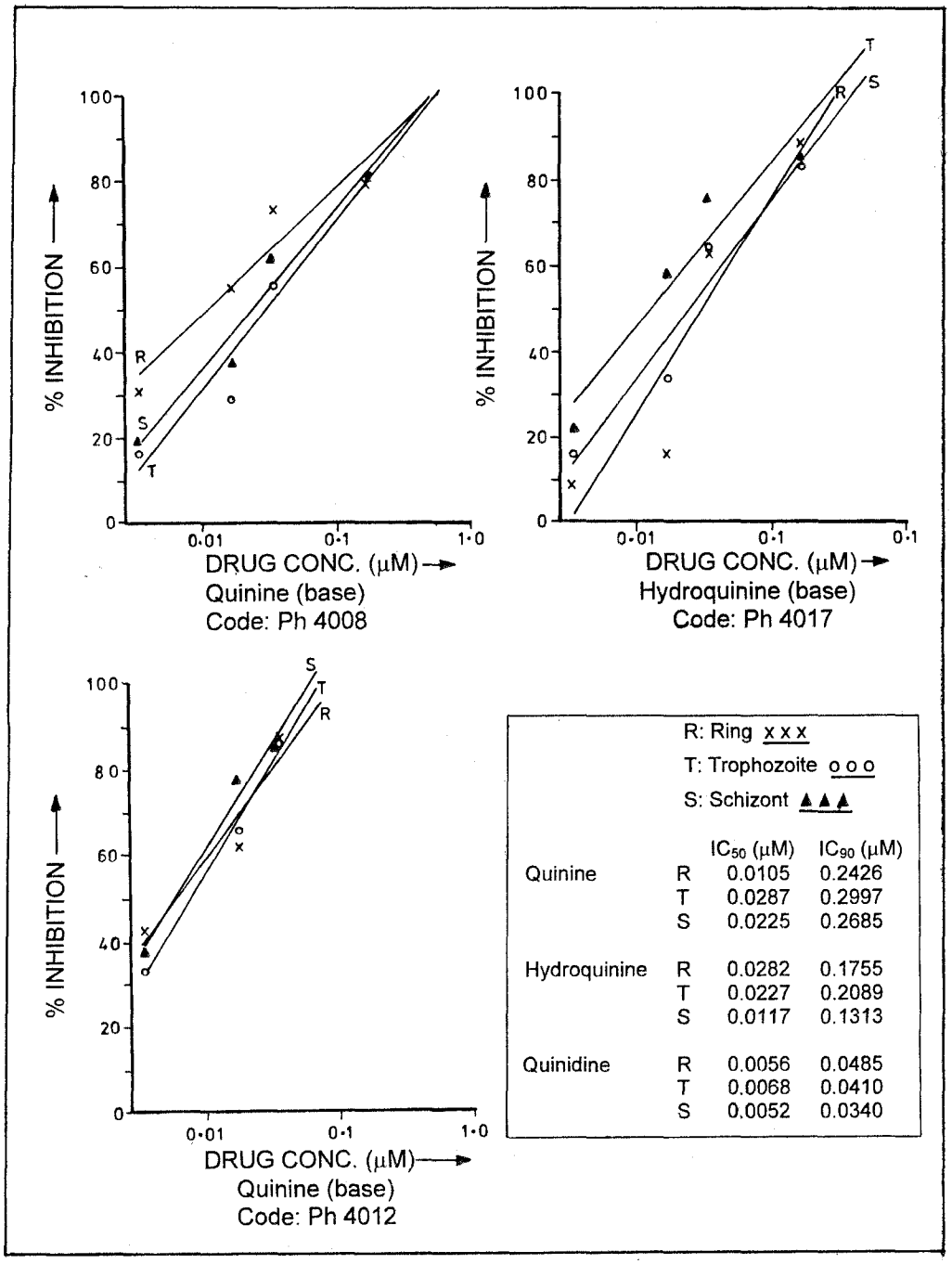


Fig. 4: Stage-specific effect of the antimalarials (quinine, hydroquinine, quinidine) in synchronous cultures of the parasite isolate

parasite as the pigment is more in these stages, thereby forming a chloroquine-ferriprotoporphyrin complex. The drug reaches millimolar concentrations in the food vacuole of a susceptible trophozoite and possibly inhibits the enzyme haem polymerase, thereby disrupting the ordered conversion of haemoglobin bound haem into haemozoine[8]. Haem is highly toxic for malarial protease[9] and would, therefore, rapidly block further haemoglobin degradation. Quinine, being similar to chloroquine in its structure, could also have similar mode of action, as both bind with high affinity to the same receptor[2]. The toxic effects of quinine and similar drugs on parasites may relate to the rapid solubility of the hemichrome coordination complex. However, quinidine, like epiquinine, may not form such a complex[10], therefore, the dug probably acts by accumulating in the vacuole and thereby inhibiting the enzyme haem polymerase.

The present data thus demonstrate that the drugs quinine, hydroquinine, quinidine and the metabolites of chloroquine SA100487/053B were lethal in short exposure without particularly being stage-specific. But chloroquine and its other metabolite SA100387/050B were less potent and more effective against the mature stages of the parasite.

ACKNOWLEDGEMENTS

This investigation received financial support from the University Grants Commission, India. We thank Dr. Peter Buchs for synthesizing these compounds.

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Variations in the Response to *Bacillus sphaericus* Toxins in Different Strains of *Anopheles stephensi* Liston

P.K. MITTAL, T. ADAK and V.P. SHARMA

Bacillus sphaericus has a potential of use as a larvicide in water storage practices but no such study has been done against *Anopheles stephensi*. Baseline susceptibility status of eleven wild and three mutant strains of *An. stephensi* to Spherix, a formulation of *B. sphaericus*, strain B-101 (serotype H5a, 5b) was determined to find the variations in the degree of their response to *B. sphaericus* toxins and to study the possibility of development of resistance. The LC_{50} and LC_{90} values of different strains of *An. stephensi* to *B. sphaericus* formulation varied from 0.088 to 1.42 mg/l and 0.314 to 10.98 mg/l, respectively. Among all the strains tested, Sarojini Nagar, Delhi strain of *An. stephensi* (a wild type strain) was least susceptible. Laboratory selection of Sarojini Nagar strain of *An. stephensi* with *B. sphaericus* at a concentration of 4 mg/l resulted in the development of a high degree of resistance (LC_{50} > 1600 mg/l) to *B. sphaericus* within four generations.

Keywords: *Anopheles stephensi, Bacillus sphaericus*, Resistance, Variations

INTRODUCTION

Anopheles stephensi Liston type form is a major vector of urban malaria in India[1]. This species essentially breeds in contained water habitats such as overhead tanks, water storage at construction sites, piped water leakages in sluice valve chambers, ornamental fountain tanks, cemented pools, wells etc[2]. Outbreaks of malaria in urban areas due to *An. stephensi* at the construction sites have been reported[3,4]. In India control of urban malaria is carried out under urban malaria scheme (UMS) which is based on anti-larval methods. At present about 46 million popu-

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lation living in 29 cities is under high risk requiring accelerated urban malaria control programme. Presently malariol oil, temephos and fenthion are being commonly used in anti-larval operations but all these compounds are also toxic to co-existing beneficial organisms. As an alternative to these chemicals, *B. thuringiensis israelensis* H-14 (Bti) and *B. sphaericus* are emerging as eco-friendly anti-larval agents. Some formulations of these microbial agents have been found effective for the control of *Cx. quinquefasciatus, Cx. tritaeniorhynchus* and *An. stephensi*[5-7]. Spraying of *B. sphaericus* (strain B-101) to control mosquito breeding under field conditions was shown to cause development of resistance in field populations of *Cx. quinquefasciatus*[8,9]. But resistance to *B. sphaericus* in *An. stephensi* has not been reported so far. The present study was undertaken with the main objective to find the possibility of the development of resistance to *B. sphaericus* toxins in *An. stephensi*.

MATERIALS AND METHODS

The following *An. stephensi* strains being maintained and colonised at the Malaria Research Centre were used in the present study: *Wild strains*: (1) Fezerpura, Madhya Pradesh (Fz); (2) Alwar, Rajasthan (Al); (3) Jabalpur, Madhya Pradesh (Jb); (4) Rithala, Delhi (Ri); (5) Madras, Tamil Nadu (Md); (6) Sonepat, Haryana, (So); (7) Hoshiarpur, Punjab (Hp); (8) Nehru Place, New Delhi (NP); (9) Sonapur, Assam (Sp); (10) Panaji, Goa (Pn); and (11) Sarojini Nagar, New Delhi (SN); *Mutant strains*: (1) Black larva (*Bl*), an autosomal dominant mutant[10]; (2) Golden-yellow larva (*Gy*), an autosomal recessive mutant[10]; and (3) Red eye (*Re*), a sex linked mutant strain[11].

Spherix, a formulation of *B. sphaericus* (B-101), serotype H5a, 5b developed in Russia and supplied by Biotech International Ltd., Delhi was used in this study.

Susceptibility status of different strains of *An. stephensi* to *B. sphaericus* was determined using III instar larvae. Total eight serial concentrations of *B. sphaericus,* viz. 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/l and a control were used in the bioassays. These concentrations were obtained by adding one ml suspension of *B. sphaericus* formulation (serial dilution made from an original 0.1% stock suspension in water) in 249 ml water. The different dilutions of *B. sphaericus* were used after thoroughly mixing the suspension with a magnetic stirrer for about two min. Twenty-five larvae were exposed to a final volume of 250 ml water. There were two replicates for each concentration and the control, and each test was repeated twice.

All the tests were carried out at $28 \pm 2^{\circ}C$, to avoid effect of temperature variation on biocide activity[12]. The mortality of larvae was recorded after 48 h of exposure, and dose-mortality regression was done by log-probit method[13]. Among all the strains tested, Sarojini Nagar strain (SN) was found to be least susceptible to *B. sphaericus*. Subse-

quently this strain was subjected to selection pressure for the development of resistance. Late III instar larvae of this strain were exposed to a dose equivalent to 4 mg/l of *B. sphaericus* (as this dose produced almost 100% mortality in most of the strains of *An. stephensi*) for 48 h. Thereafter, surviving larvae were separated, washed and reared into adults to obtain F_1 generation. The larvae obtained from F_1 adults were again treated at 4 mg/l of *B. sphaericus* and the surviving larvae were reared into adults to obtain F_2 generation. This procedure was continued for selection of resistance in the subsequent generations up to fifth generation. Larvae from each generation of the selected Sarojini Nagar strain were also tested for susceptibility to *B. sphaericus*.

RESULTS

Dose-mortality responses of different strains

Table 1. Dose-mortality responses of different *Anopheles stephensi* strains against *Bacillus sphaericus*

An. stephensi strains	Concentration (mg/l)			
	LC_{50}(95% fiducia limits)	LC_{90}(95% fiducial limits)	Slope	χ^2 (df)
Wild strains				
Fz–Fezerpura (M.P.)	0.147 (0.165-0.131)	0.46 (0.547-0.385)	2.59	9.799(6)
Al–Alwar (Rajasthan)	0.228 (0.252-0.206)	0.544 (0.631-0.468)	3.4	2.35(6)
Jb–Jabalpur (M.P.)	0.257 (0.291-0.226)	0.994 (1.202-0.814)	2.19	4.96(6)
Rl–Rithala (Delhi)	0.164 (0.196-0.137)	0.647 (0.961-0.435)	2.15	5.60(6)
Md–Madras (T. Nadu)	0.211 (0.258-0.173)	0.957 (1.341-0.682)	1.95	3.10(6)
So–Sonepat (Haryana)	0.269 (0.309-0.234)	1.40 (1.75-1.115)	1.79	5.01(6)
Hp–Hoshiarpur (Punjab)	0.264 (0.307-0.227)	1.64 (2.1-1.28)	1.61	32.06(6)
NP–Nehru Place (New Delhi)	0.322 (0.374-0.28)	1.869 (2.37-1.45)	1.68	10.78(6)
Sp–Sonapur (Assam)	0.232 (0.273-0.197)	1.70 (2.221-1.499)	1.48	37.13(6)
Pn–Panaji (Goa)	0.296 (0.345-0.254)	1.88 (2.43-1.46)	1.6	13.03(6)
SN–Sarojini Nagar (New Delhi)	1.42 (1.725-1.169)	10.98 (16.52-7.278)	1.45	4.43(6)
Mutants				
Red eye (*Re*)	0.101 (0.116-0.088)	0.409 (0.499-0.33)	2.11	12.43(6)
Golden yellow (*Gy*)	0.088 (0.107-0.073)	0.314 (0.418-0.236)	2.325	1.75(6)
Black larvae (*Bl*)	0.529 (0.661-0.423)	10.39 (17.66-6.11)	0.995	33.75(6)

of *An. stephensi* against *B. sphaericus* formulation are shown in Table 1. The LC_{50} values of different strains ranged between 0.088 and 1.42 mg/l, while LC_{90} between 0.314 and 10.98 mg/l. Among all wild strains, Fezerpura strain was most susceptible (LC_{50} and LC_{90} = 0.147 and 0.46 mg/l, respectively), while Sarojini Nagar strain was least susceptible (LC_{50} and LC_{90} = 1.42 and 10.98 mg/l respectively). There was a significant difference in the LC_{50} and LC_{90} values for Sarojini Nagar strain as well as for the rest of the wild strains as there was no overlapping of 95% fiducial limits. The LC_{50} value of Sarojini Nagar strain was 4.4 to 12.5 times higher and LC_{90} value was 5.8 to 23.8 times higher than the respective values for other wild strains tested.

Among the three mutant strains, Red eye (*Re*) and Golden yellow larvae (*Gy*) were highly susceptible to *B. sphaericus* (LC_{50} = 0.10 and 0.088 mg/l; and LC_{90} = 0.41 and 0.31 mg/l respectively), while Black larvae (*Bl*) were relatively tolerant (LC_{50} and LC_{90} values being 0.53 and 10.39 mg/l respectively).

Selection pressure with *B. sphaericus* at a dose of 4 mg/l resulted in the development of a very high degree of resistance in Sarojini Nagar strain within four generations (Table 2). The LC_{50} value estimated for the larvae of F_1, F_2, F_3 and F_4 generations were 17.9, 32.6, 330 and >1600 mg/l respectively, which were approximately 13 to 1100 times higher than the initial value estimated for the parental strain. No mortality was observed in F_5 generation larvae with 4 mg/l dose and LC_{50} value was > 1600 mg/l.

Table 2. Data showing development of resistance in *An. stephensi* against *Bacillus sphaericus* during laboratory selection

Generation of mosquito	LC_{50} (mg/l)	Resistance ratio*
P–Parental	1.42	—
F_1–Selected	17.91	12.6
F_2–Selected	32.59	22.95
F_3–Selected	~ 330.00	~ 232.4
F_4–Selected	> 1600.00	>1126

$$*\text{Resistance ratio} = \frac{LC_{50}\text{(Selected generation)}}{LC_{50}\text{(Parental generation)}}$$

DISCUSSION

B. sphaericus and *B. thuringiensis* var. *israelensis*, the two microbial agents have been shown as promising alternatives to conventional larvicides in some preliminary trials against urban mosquito vectors in India. However, in a multicentric field trial continuous application of *B. sphaericus* at fortnightly interval, for one year, revealed the development of *B. sphaericus* resistance in field populations of *Cx. quinquefasciatus*[8]. Nielsen-Leroux *et al.*[14] reported that the resistance in *Cx. quinquefasciatus* to *B. sphaericus* binary toxin was due to a change in the receptors in the larval midgut brush-border membranes in the resistant strain. The binary toxin of *B. sphaericus*, which con-

sists of 51 and 42 kDa proteins, require a single class of receptors for binding. One of these toxins helps in binding to the receptor sites and the other performs toxic action[15].

Although various reports on resistance in *Cx. quinquefasciatus* to *B. sphaericus* toxin have been published, there is no such report against *An. stephensi*. The results of the present study have shown the occurrence of natural variations in response to *B. sphaericus* among different strains of *An. stephensi* and also the development of a high level of *B. sphaericus* resistance through laboratory selection in Sarojini Nagar (SN) strain of *An. stephensi*. It is evident that different strains of *An. stephensi* (except Sarojini Nagar wild strain and black larvae mutant strain) were highly susceptible to *B. sphaericus* and showed almost 100% mortality at a dose of 4 mg/l. However, the same dose produced only about 80% mortality in Sarojini Nagar strain, indicating that this strain perhaps consisted of a mixed population of *B. sphaericus* susceptible and resistant genotypes. When this dose (4 mg/l), which produced almost 100% mortality in most susceptible strains, was used for selection of *B. sphaericus* resistance in Sarojini Nagar strain of *An. stephensi*, it resulted in the development of highly resistant strain. In an earlier study resistance to *B. sphaericus* in *Cx. quinquefasciatus* was found to be a recessive character[8]. This seems perhaps true for *An. stephensi* also, as the laboratory selection of Sarojini Nagar strain with a dose of 4 mg/l of *B. sphaericus* might have rapidly eliminated most of the susceptible homozygote and heterozygote populations and subsequently established homozygocity for *B. sphaericus* resistance within four generations. Although development of resistance was very fast in this strain of *An. stephensi* in the present study, the development of resistance in wild populations will, however, depend on the frequency of pre-selected *B. sphaericus* resistant genes and the selection pressure. Under the present circumstances, *B. thuringiensis israelensis* seems to be a better alternative than *B. sphaericus* as there is a lesser possibility of development of resistance against its toxin[16].

Although Dai and Gill[17] reported about 70 fold resistance to a purified 72 kDa Bti toxin in *Cx. quinquefasciatus*, only a three fold resistance to whole crystal-endotoxin preparation of Bti was detected. In contrast to binary toxin of *B. sphaericus*, which require a single class of receptors for binding[14], there are four mosquitocidal endotoxins in Bti which are synergistic in action and have a different mechanism of cell membrane interaction[18]. Individually none of these toxins are as toxic as the spore-crystal complex and therefore, the chances of development of resistance to Bti are lower[18]. In order to achieve the maximum advantage of two larvicidal agents, formulations could be developed by combining both, *B. sphaericus* and Bti toxins which would delay the development of resistance and also provide broad spectrum effect for a longer duration.

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Impact of Spherix (*Bacillus sphaericus* B-101, Serotype H5a, 5b) Spraying on the Control of Mosquito Breeding in Rural Areas of Farrukhabad District, Uttar Pradesh

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Spray impact of Spherix (*Bacillus sphaericus* B-101, Serotype H5a, 5b) was assessed against larval and adult populations of mosquito species in rural areas of Farrukhabad district, Uttar Pradesh. High mortality (> 95%) was observed against anopheline and culicine larvae within 48 h of spray @ 1 g/sq m. But the biolarvicidal effect declined within a week which indicated non-recycling capacity and weekly reapplications were required. The encouraging results obtained through weekly sprays were short-lived because of decline in efficacy in some perennial intradomestic Culex breedings after 15 rounds of spray. The two year intensive field trials, however, had no appreciable impact on adult densities of both malaria vector *An. culicifacies* and bancroftian filariasis vector *Culex quinquefasciatus*. Environmental disturbances and man-made problems also affected the efficacy of the biolarvicide.

Keywords: Mosquito, Spherix

INTRODUCTION

Under the National Malaria Eradication Programme (NMEP) residual insecticides are sprayed indoors for interrupting malaria transmission in rural areas. But due to technical, operational and environmental problems the conventional control methods failed to contain malaria. This resulted in focal outbreak of malaria in areas under insecticide spray[1]. At present environmental friendly technology is needed to combat malaria. Emphasis

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is laid on biological control of disease vectors with larvivorous fish and biolarvicides. Among the entomopathogenic organisms some strains of *Bacillus sphaericus* were found promising biolarvicide[2-4]. Extensive multicentric field trials carried out by Malaria Research Centre[5] in India showed that the Spherix spray (a commercial preparation of *B. sphaericus*, Russian strain B-101, Serotype H5a, 5b) @ 1 g/sq m provides control of bancroftian filariasis vector *Cx. quinquefasciatus* breeding for 2-4 wk. All the multicentric trials were made either in a limited area or in urban situations. No extensive field trials had been done for the control of rural malaria vector *An. culicifacies*. Therefore, Spherix was applied in different types of mosquito breeding sites of 32 villages of Talgram Primary Health Centre (PHC) of Farrukhabad district with the aim to study the impact of large-scale field applications of Spherix in controlling mosquitoes in rural areas and development of resistance, if any during the long course of applications. Results of the study collected from March 1993 to February 1995 (two years) are communicated in this paper.

MATERIALS AND METHODS

Study area

District health authorities of Farrukhabad district, Uttar Pradesh observed abnormal rise in malaria incidence in some villages of Talgram Primary Health Centre (PHC) during the post-monsoon season of 1991. A total of 32 such villages under Gursahaiganj sub-PHC were undertaken for biolarvicidal spray with Spherix. The entire set of villages are located within 20 km radius southwest of Gursahaiganj town situated on the historic Grand Trunk (GT) Road 325 km east of Delhi and 105 km west of Kanpur. The residual insecticidal spray was withdrawn from the experimental villages during the present trial. Two villages situated beyond three km from the experimental villages were taken as control. DDT spray under state NMEP remained in operation in the control villages.

Population census and larval surveys

Before application of biolarvicide, village-wise information on population, geographical reconnaissance (GR) for mapping mosquito breeding potentials, adult mosquito densities and parasite incidence were collected during February to March 1993. Census revealed human population 18,889 and cattle was 10,465 and the ratio of cattle to human population was 1:1.8.

As per GR larval habitats were categorised into domestic and peri-domestic breeding sites. The domestic breeding sites were water collections in pits, ditches, pitchers, street drains, tanks, waste water near community taps and wells. Almost every household had dug a pit in the house for cleaning used kitchenwares. Such pits locally known as *Nandhs* (trough) were never emptied and thus formed the perennial breeding grounds for *Cx. quinquefasciatus*. Due to improper drainage in the entire area heavy stagnation

of waste water supported breeding of *Culex* mosquitoes. Surface area of the domestic breeding sites was approximately 5,000 sq m. The peridomestic breeding sites consisted of temporary and permanent ponds, water collections in depressions, seepage from tube well irrigation channels, cold storage tanks, roadside borrowpits and seasonal drains. Area of above breeding sites was approximately 30,000 sq m.

There is no river or irrigation canal in the selected villages. However, extensive irrigation is done through tube wells. On an average there are 15 tube wells per village. Each tube well has water tank which supported mosquito breeding if water is stored for a longer period. During the survey 320 wells out of 400 searched were found dry due to low level of underground water table. The terrain was plain Gangetic with sandy soil having high water absorption capacity. Average annual rainfall during 1993 and 1994 was 452.5 mm. Number of rainy days were 37 and 45 during the above years respectively. Only small precipitation occurred in the beginning of the year. Temperature ranges from 6 to 45°C and May and June are the hottest months of the year. Xerophytic plants like Tar and Cactus are found in the area.

The economic activities of the people are farming and bidi making. Truck loads of tendu leaves are brought from Madhya Pradesh (Satna district) for *bidi* wrapping.

The major crops grown in the area are potato, sunflower, wheat, maize, ground nut pods and pulses. The potato and sunflower crops require 5-6 times watering for maturation. This resulted in high spillage of water from tube wells and thus aggravating the mosquito problems. Rice and sugarcane are cultivated only in a limited area.

Application of biolarvicide and larval monitoring

Spherix was sprayed @ 1 g/sq m on the margins of all the breeding sites with the help of knapsack sprayer having flat fan nozzle. Biolarvicide was sprayed weekly due to reappearance of III and IV instar larvae in the treated habitats. No applications were done in potable water and wells. However, Gambusia fish were released in wells so as to ensure check on mosquito larvae. No spray was done on rainy days and during winter months (December to February) due to low temperature. To assess spray impact larval densities were monitored weekly in eight indicator villages. The weekly data collected was pooled month-wise. For a comparative study larval densities in control habitats were monitored fortnightly upto 12 wks thereafter, weekly as in the experimental habitats.

The total quantity of Spherix sprayed during the present trials was 1,466.918 kg.

Before treatment approximate surface area of each habitat was measured after which the amount of biolarvicide was weighed and mixed with water. The results were compared with those of unsprayed habitats of control villages.

Table 1. Impact of Spherix (*Bacillus sphaericus*) spray on the immature densities of anopheline and culicine mosquitoes from March 1993 to February 1995

Observation Day/Month/Year	Anopheline				Culicine			
	III and IV larvae		Pupae		III and IV larvae		Pupae	
	Exp	Cont	Exp	Cont	Exp	Cont	Exp	Cont
March 1993								
Day 0 (before treatment)	5.30	0.90	0.20	0.10	178.90	59.6	21.10	6.80
Day 1 (after treatment)	0.20	*	0.10		52.50	*	12.80	
Day 3 (after treatment)	0.50	*	0.20		45.80	*	9.0	
Day 5 (after treatment)	0.50	*	0.10		31.90	*	7.80	
Day 7 (after treatment)	0.90	*	0.00		2.30	*	0.30	
April 1993	0.50	1.60	0.00	0.30	0.90	47.8	0.07	5.50
May	1.10	3.70	0.03	0.50	0.80	48.7	0.00	10.50
Jun	0.70	4.20	0.00	0.50	0.50	22.4	0.00	5.70
Jul	0.50	3.20	0.01	0.40	0.50	4.8	0.04	0.80
Aug	0.20	0.90	0.00	0.06	0.30	5.7	0.01	0.90
Sep	0.10	1.10	0.00	0.08	0.10	4.4	0.01	0.60
Oct	0.08	1.00	0.00	0.10	0.20	6.5	0.00	1.20
Nov	0.20	0.90	0.00	0.04	0.30	12.2	0.01	3.10
Dec	0.20	0.40	0.01	0.06	1.50	30.9	0.40	9.60
Jan 1994	0.09	0.30	0.00	0.02	2.90	31.8	0.80	10.40
Feb	0.08	0.14	0.01	0.02	2.20	35.0	0.90	17.90
Mar	0.10	0.34	0.00	0.02	3.70	61.5	0.95	18.50
Apr	0.16	0.50	0.00	0.10	11.06	62.9	3.02	20.50
May	0.20	0.90	0.00	0.10	3.00	52.4	0.50	18.80
Jun	0.14	1.30	0.00	0.14	0.60	18.5	0.10	5.50
Jul	0.13	0.94	0.01	0.12	0.70	2.3	0.10	0.40
Aug	0.10	0.54	0.00	0.10	0.40	2.7	0.04	0.42
Sep	0.10	0.40	0.00	0.02	0.50	2.5	0.10	0.40
Oct	0.14	0.23	0.00	0.01	1.30	7.3	0.20	1.80
Nov	0.30	0.50	0.01	0.02	2.20	36.5	0.70	5.50
Dec	0.30	0.30	0.02	0.01	9.50	36.2	2.00	9.20
Jan 1995	0.10	0.10	0.01	0.00	5.00	15.1	1.80	4.50
Feb	0.10	0.10	0.01	0.01	3.10	31.9	1.00	11.90

*Immature density in control was not recorded on Day 1, 3, 5 and 7 after treatment.

A standard dipper was used to estimate the immature densities by taking 5 dips in pits measuring < 5 sq m, 10 dips in pits with 5-10 sq m and 20 dips in sites with area >10 sq m. The per cent reduction in the density of larvae was calculated using Mulla's formula[6] given below:

$$\% \text{ reduction} = 100 - \frac{C_1}{T_1} \times \frac{T_2}{C_2} \times 100$$

Where, C_1 = No. of larvae in control pre-treatment; C_2 = No. of larvae in control post-treatment; T_1 = No. of larvae in treated pre-treatment; and T_2 = No. of larvae in treated post-treatment.

Adult mosquito density

To assess impact of Spherix spray on mosquito densities adult hand collections were carried out fortnightly by suction tube method. Four villages two each in experimental and control were fixed for monitoring mosquito density. Fortnightly densities were pooled month-wise. For impact assessment adult densities were categorised into total mosquitoes, anophelines, culicines and vectors.

RESULTS AND DISCUSSION

Results of field applications of spherix are discussed below:

Larval impact: The impact was recorded against both anopheline and culicine larvae.

Impact on anopheline larvae

Anopheline larval density recorded weekly in ponds, pools, pits, drains and ditches was pooled monthly and given in Table 1. The average larval density before treatment (Day 0) was recorded 5.3 per dip. On Day 1, 3, 5 and 7 post applications it declined to 0.2, 0.5, 0.5 and 0.9 per dip respectively. Pupal density declined from 0.2 (Day 0) to 0.1, 0.2, 0.1 and 0.0 on 1, 3, 5 and 7 day of post-treatment respectively. Further monitoring revealed low immature densities in experimental as compared to control habitats. But the larval density was not decimated in the experimental habitats.

However, in the first week of May 1993 there was a sudden increase in anopheline larval density due to profuse breeding of *An. subpictus* (seasonal mosquito) in pits and ponds. This happened immediately after the first precipitation of the rainy season. During May and June water level in ponds and pits reduces considerably. Villagers dig out mud for plastering their houses before onset of rainy season. Under such circumstances Spherix applied in ponds and pits was taken away alongwith the mud. This resulted in less impact of the spray on mosquito breeding. This operational difficulty (man-made problem) is a common feature in rural areas. During summer season though water level in village ponds reduced but its average surface area increased due to its division into several pits. Removal of biolarvicide particles along with mud reduced its larvicidal

property and thus resulted in less impact. In this regard our observations were similar to those reported by Mulla[7].

Impact on culicine larvae

Average larval density in various breeding habitats before application (Day 0) was found 178.9 per dip. On Day 1, 3, 5 and 7 post application days it reduced to 52.5, 45.8, 31.9 and 2.3 per dip respectively (Table 1). Further monitoring revealed low densities in experimental as compared to control sites. However, a slight increase in larval density was observed during December onwards. This may be due to the reason that Spherix spray was stopped during winter months (December to February) due to unfavourable season for malaria transmission and low temperature. However, small-scale field trials were carried out to see its impact during winter months. The average maximum temperature during December and January ranged from 18.3 to 19.0°C. Results of such trials are given in Table 2. It was observed that on Day 1 after treatment Spherix produced 25.6 and 58.1% mortality of culicine larvae even in winter months of 1993 and 1994 respectively. However, the present mortality was slightly lesser than that obtained during summer season. Variations in larvicidal activity of *Bacillus sphaericus* due to physical parameters of the environment were also reported by Mulla[7] and Mittal[4]. The increased larvicidal activity during summer may probably be due to the increased metabolic activ-

Table 2. Impact of Spherix (*Bacillus sphaericus*) spray on culicine larvae during winter season (Average temperature 18.3 to 19.0°C)

Observation (Day)	Period of trial					
	13.12.1993 to 20.12.1993			24.01.1994 to 31.01.1994		
	Density/Dip		% mortality (III and IV instar)	Density/Dip		% mortality (III and IV instar)
	Exp	Cont		Exp	Cont	
Before treatment						
Day 0	276.3	103.9	0.0	88.0	60.8	0.0
After treatment						
Day 1	187.3	168.2	58.1	96.8	89.9	25.6
Day 2	161.7	151.0	59.7	39.0	290.8	90.7
Day 3	110.0	150.7	72.6	1.7	193.6	99.4
Day 5	3.0	155.0	99.3	0.2	174.6	99.9
Day 7	1.5	156.4	99.6	23.3	101.0	84.1

Table 3. Impact of Spherix (*Bacillus sphaericus*) spray on culicine larvae in repeatedly sprayed old pits and fresh pits

Observation (Day)	Larval density (III and IV instar) per dip					
	Repeatedly sprayed pits after 105 days of post-treatment			Fresh pits		
	Exp	Cont	%mortality	Exp	Cont	%mortality
Before treatment						
Day 0	284.7	143.0	0.0	10.6	13.2	0.0
After treatment						
Day 1	290.0	145.0	0.0	9.8	13.7	11.0
Day 2	322.0	155.0	0.0	0.5	14.3	95.6
Day 3	314.0	163.0	3.2	0.3	22.3	98.3
Day 5	380.0	174.0	0.0	0.3	10.8	96.5
Day 7	327.0	190.0	13.6	0.1	27.7	99.6

ity of the larvae resulting in rapid intake of deltatoxin produced by Spherix in the larval gut. The larvicidal activity persisted for different durations in different habitats that could be due to factors like organic pollution, high water pH and environmental disturbances as discussed under anopheline larval impact. Variations in susceptibility of mosquito larvae to the same formulation of *B. sphaericus* was also observed by Mittal *et al*[8].

As pointed out by Mulla[7] the microbial control agents like *Bacillus sphaericus* and *Bacillus thuringiensis* provided immediate short-term control of larvae. Non-recycling capacity of Spherix was observed separately during the present investigations. WHO[3] and Bihari *et al.*[2], however, have earlier reported recyling capacity in some strains of *Bacillus sphaericus*.

The close monitoring of field data revealed that after XV round of weekly spray impact on culicine larvae declined in some intradomestic sites. Larval monitoring in such pits was done separately for a week and results are given in Table 3 which indicated nil to very low (3.2%) mortality in repeatedly sprayed pits upto Day 5 after treatment. Whereas mortality in fresh pits was 95.6% after 48 h of treatment. Part of the reasons for low mortality may be due to the fact that intradomestic pits are the perennial breeding sites for *Culex* mosquitoes. Stagnant pools of water either intradomestic or in the streets are found throughout the

Table 4. Per man hour densities of mosquitoes in Spherix (*Bacillus sphaericus*) sprayed and control areas of Farrukhabad district from April 1993 to March 1995

Month/Year	Mosquito		Anopheline		Culicine		Vector	
	Exp	Cont	Exp	Cont	Exp	Cont	Exp	Cont
April 1993	108.5	113.0	47.0	10.5	61.5	102.5	1.0	4.0
May	22.5	80.8	15.0	37.5	7.5	43.3	0.5	0.3
Jun	38.6	82.1	25.8	34.3	12.8	47.8	2.3	0.8
Jul	67.3	123.8	46.8	90.0	20.5	33.8	1.0	5.0
Aug	71.8	91.5	60.8	65.0	11.0	26.5	2.8	2.0
Sep	65.8	78.5	50.8	58.5	15.0	20.0	1.3	1.3
Oct	61.5	62.8	46.5	40.0	15.0	22.8	1.8	0.8
Nov	36.3	44.8	20.5	24.0	15.8	20.8	0.8	0.5
Dec	58.5	78.8	31.0	35.8	27.5	43.0	0.3	0.5
Jan 1994	29.0	36.3	3.5	4.5	25.5	31.8	0.0	0.0
Feb	24.6	34.3	3.3	5.0	21.3	29.3	2.5	0.0
Mar	110.8	119.8	10.3	7.3	100.5	112.5	1.0	0.5
Apr	120.6	186.3	12.3	17.5	108.3	168.8	0.3	0.0
May	31.6	72.3	15.8	31.5	15.8	40.8	0.0	0.0
Jun	15.6	43.1	9.3	23.8	6.3	19.3	0.0	0.0
Jul	266.6	286.8	183.3	212.3	83.3	74.5	3.5	6.3
Aug	542.5	540.0	499.0	512.0	43.5	28.0	20.5	14.5
Sep	151.5	157.0	128.5	125.5	23.0	31.5	4.0	10.0
Oct	169.0	218.5	113.5	149.5	55.5	69.0	2.5	5.0
Nov	161.5	186.5	87.0	99.0	74.5	87.5	2.5	5.0
Dec	107.5	106.0	45.5	38.5	62.0	67.5	2.0	2.5
Jan 1995	39.0	30.0	5.5	6.0	33.5	24.0	0.0	0.0
Feb	99.0	110.0	30.0	2.0	69.0	108.0	2.0	0.0
Mar	182.0	218.0	12.0	12.0	170.0	206.0	0.0	0.0

Note: July-August 1993 — Rainy days 18, rainfall 112.4 mm; and July-August 1994 — Rainy days 30, rainfall 522.4 mm.

year due to improper disposal of waste water. As a result of this the other microflora in stable pits might have provided alternate food for the larvae and perhaps reduced intake of biolarvicide. This results in production of more generation, high population and thus probably more selection pressure for survival. Similar phenomenon was reported by Shelton *et al.*[9] in diamond back moth (Crucifer pest) against pesticide formulations of *Bacillus thuringiensis* sub species in Florida (USA). They pointed out that Crucifer plants were grown almost throughout the year which resulted in more generations of pest being produced, higher populations and more selection pressure for resistance. Population of *Culex quinquefasciatus* from the unsprayed and sprayed areas of Farrukhabad were transported to Delhi for the study conducted by Adak *et al.*[10] In their study it was pointed out that the LC_{50} and LC_{90} values of population from unsprayed area were significantly lower than those of the population from the sprayed area. The selection of resistance to *B. sphaericus* in field population of *Culex quinquefasciatus* was observed by them through slope values and resistance ratio (RR). Rao *et al.*[11] recorded field resistance to *Bacillus sphaericus* in population of *Cx. quinquefasciatus* exposed to 35 rounds of spraying with a formulation of *Bacillus sphaericus* 1593 in over a two-year period.

Impact of adult densities

Results of adult mosquito densities monitoring in the experimental and control areas are given in Table 4. Densities of total mosquitoes, anophelines, culicines and vector (*An. culicifacies*) in experimental varied from 15.6 to 542.5, 3.3 to 499.0, 6.3 to 170.0 and 0.0 to 20.5 respectively. The above densities in control varied from 30.0 to 540.0, 2.0 to 512.0, 19.3 to 206.0 and 0.0 to 14.5 respectively. Results revealed that during the first six months of the study (April to September) densities of total mosquitoes and anophelines remained low in experimental as compared to control. Thereafter, the densities were comparable in both the areas. Reduction in culicine densities occurred in experimental from April 1993 to February 1994. However, a similar decline was also observed in control. Further decline in densities did not occur on a definite pattern.

Except in August 1994 malaria vector densities remained low both in experimental and control areas. The higher densities in above months may be attributed to high rainfall (522.4 mm) and more number of rainy days (30) during July and August 1994 as compared to the corresponding months of 1993 in which only 112.4 mm rainfall and 18 rainy days were recorded. The low densities during other months were mainly due to less number of preferential breeding sites of *An. culicifacies*. Densities varied from month to month. No clear-cut impact of Spherix spray was observed on vector densities. Like other parts of northern India malaria in this area is transmitted during the monsoon season. Anopheline mosquitoes including malaria vector *An. culicifacies* attained high peak densities during July to October covering the mon-

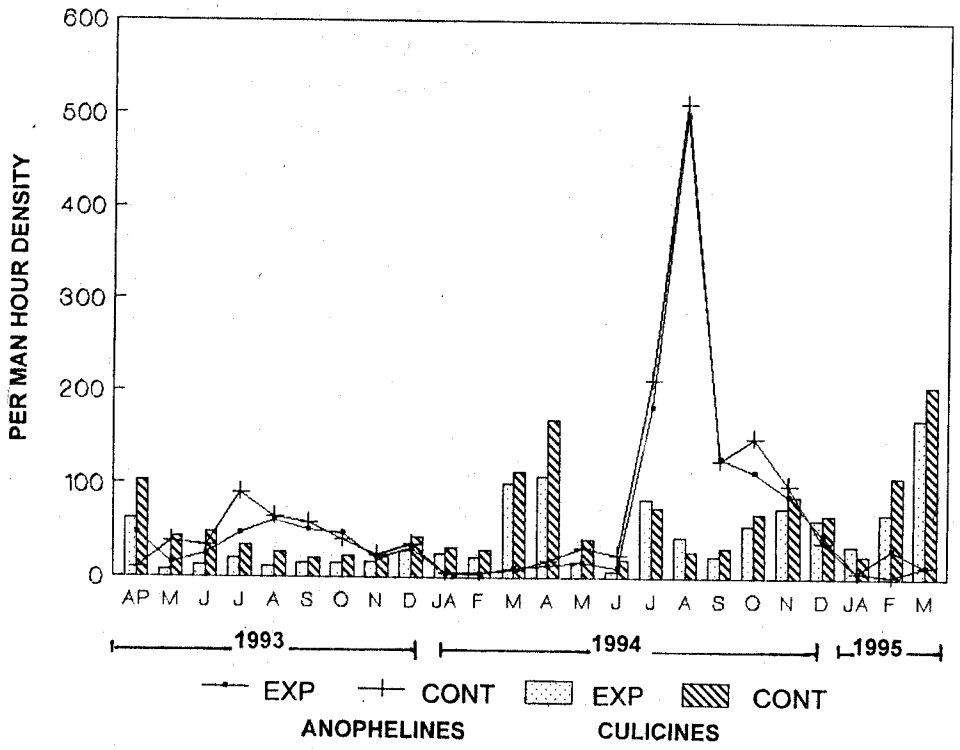

Fig. 1: Per man hour densities of anophelines and culicines in Spherix (*Bacillus sphaericus*) sprayed and control areas of Farrukhabad district from April 1993 to March 1995

soon season (Fig. 1). Part of the reason for higher densities in these months may be due to suspension of spray operation during rainy season (July-August). As a result of this *An. culicifacies* built up high densities during the above season.

Results revealed that average densities of culicine mosquitoes were higher during March to May as compared to other months of the year (Fig. 1). Due to drying of pits and less environmental disturbances like rainfall and temperature, an effective biolarvicidal spray would certainly help in curtailing culicine densities during this period. Earlier some formulations of *Bacillus sphaericus* were found highly effective against *Culex* spp[2,3]. Results collected by Kar *et al.*[12] showed significant difference in the adult densities of *Cx. quinquefasciatus* in Spherix sprayed and control areas. Two formulations of *Bacillus sphaericus* (Solvay liquid 2362 and Abbott granules 2297) were found effective against *Cx. quinquefasciatus* but ineffective against malaria vector *An. culicifacies*[13].

Prasad *et al.*[1] had reported focal outbreak of malaria in this area. But the disease was

controlled on war footing by measures adopted by state NMEP immediately after the outbreak. Results of present study indicated that there was not much reduction in adult mosquito densities despite repeated sprays with Spherix.

The biolarvicide application was not effective during rainy season which is the favourable period for rural malaria vector *An. culicifacies*. The level of reduction in densities of *Culex* spp obtained during the initial period of the study could not be adhered due to multiple factors discussed in this paper. Finally, it may be stated that it was not feasible to control malaria vector *An. culicifacies* by means of biolarvicide spray using Spherix alone. An integrated approach is suggested for the effective control of vector mosquitoes.

ACKNOWLEDGEMENTS

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Mosquito Fauna and Breeding Habitats of Anophelines in Car Nicobar Island, India

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Atotal of 31 species of mosquitoes belonging to 10 genera, i.e. *Anopheles, Aedes, Armigeres, Culex, Harpagomyia, Hodgesia, Mansonia, Orthopodomyia, Toxorhynchites* and *Uranotaenia* were collected from Car Nicobar Island. Four *Anopheles* species, *An. barbumbrosus, An. insulaeflorum, An. kochi* and *An. roperi* were recorded for the first time from this Island. *An. sundaicus* was the most predominant species encountered. The results of the study on anophelines with emphasis on species-specific breeding preference in various aquatic habitats have been recorded.

Keywords: Car Nicobar, Fauna, Mosquitoes

INTRODUCTION

Our knowledge of mosquito fauna of Car Nicobar Island is mainly based on the work done by Basu[1]; and Krishnan and Bhatnagar[2]. With the launching of National Malaria Eradication Programme (NMEP) in the Island in 1962-63 and the availability of DDT as residual insecticide, all emphasis was placed on the control of malaria. However, for about three decades, almost no information was collected on the mosquito fauna of Car Nicobar Island. During this period there have been vast ecological changes in the fragile ecosystem. Large areas have been deforested and construction of new roads and building have come up in the Indian Air Force (IAF) and civil areas leading to a large number of new settlements. In addition to this, inter island transportation (ship services) has increased several

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times resulting in the population migration in Car Nicobar Island.

In spite of all the efforts to control malaria by the NMEP in this Island, malaria continues to be a serious problem. In view of the seriousness of the malaria situation, an alternative to chemical control method namely, bioenvironmental control strategy was launched by the Malaria Research Centre at Car Nicobar Island in the year 1989 to control malaria. In 1989 the API was 194.4 per 1000 population. This Centre also took-up research on the control of filariasis.

As a result of this, mosquito faunastic studies were conducted from January 1994 to February 1995 to collect the latest information and update the mosquito fauna of Car Nicobar Island. The results of these studies are reported in this paper.

MATERIALS AND METHODS

Car Nicobar is a small flat Island situated in the southeast corner at 6-10°C North latitude and 92-94° East longitude in the Bay of Bengal, with an area of 127 sq km (Fig. 1). The Island is made up of corals. There are seven live creeks of which Kimius creek is about 1200 ha. The western coast is covered by lush green *Barringtonia speciosa*. There is thick growth of mangroves in and around the Kimius creek. The Island has a slight elevation towards its centre which rises to about 70 m in the northwest near Passa creek. There are few small streams emerging in northern and southern regions inside the jungle of the Island which are influenced by tides. In addition to the streams, there are several water bodies and marshy areas created during monsoon which

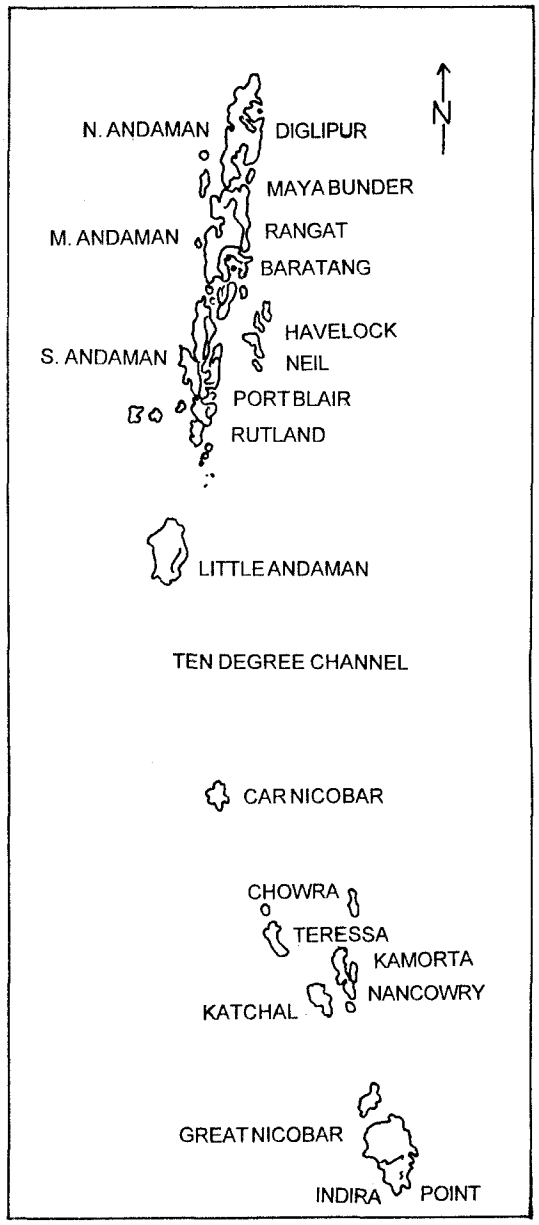

Fig. 1: Map of A&N Island showing location of Car Nicobar Island

remain up to nine months after the rainfall. There are countless coconut pits inside the coconut gardens which cover about 3 to 4 km coastal belt around the Island. About 60 per cent of the Island is covered with forest. The Island situated in the tropics has hot climate and humid weather. The temperature varies from 25 to 32°C and relative humidity from 70 to 90%. The Island receives both southwest monsoon from May to October and north-east monsoon from November to April. The annual rainfall varies from 2500 to 4000 mm. However, the rainfall is very less from January to April. The climate of Car Nicobar Island provides an ideal environment for mosquito breeding and proliferation due to high rainfall associated with hot and humid conditions.

Creeks, marshy area, mangroves, streams and wells are the perennial mosquito breeding sources. In addition to this cement tanks, ornamental tanks and ponds also support mosquito breeding.

The inhabitants of this Island are Nicobarese who are of Mongoloid origin, who live in huts made of wood, bamboo and leaves. A large number of pigs are domesticated by the Nicobarese and they are normally kept in the huts. Collection of adult and immature mosquitoes were made from January 1994 to February 1995. Adult mosquito collections were made fortnightly from eight villages, i.e. four villages each from creeky and non-creeky areas of the Island. In each village four human dwellings, four copra machans and four cattlesheds were searched. The adult mosquitoes were collected by suction tube method both during dawn and dusk. In addition mosquitoes were collected fortnightly from keori bushes, uprooted coconut stumps, tree holes, mangrove roots, croten plants and crab holes etc.

All possible larval breeding sites were searched and larvae and pupae were collected with the help of a dipper (9.5 cm diam and 300 ml capacity). Larvae and pupae were also collected by pasture pipettes from creeks, streams, ponds, mangrove areas, cement tanks, tree holes, canoes, crab holes, coral cavities, tyres, coconut shells and containers etc. The collection sites and breeding sites of mosquitoes are shown in Fig. 2 and Table 1.

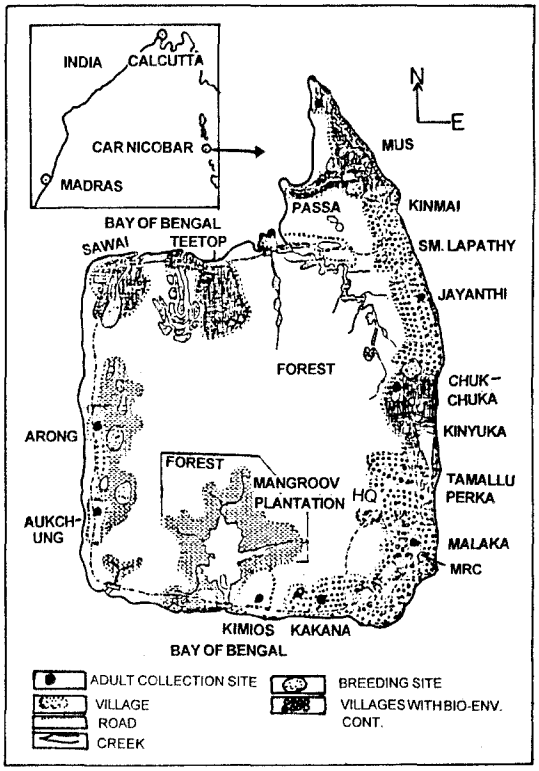

Fig. 2: Topographical map of Car Nicobar Island showing adult mosquito collection and breeding sites

Table 1. Adult mosquitoes collection sites in Car Nicobar Island

Species collected	Mosquito collection sites/structures								
	Indoor			Outdoor					
	Copra machans	Cattle-sheds	Human dwellings	Pandan-uslarum	Uprooted coconut stumps/ leaves	Coconut tree holes	Mangrove roots	Croten plants	Crab holes
An. barbirostris	+	+	–	+	–	–	–	–	–
An. barbumbrosus	+	+	–	+	–	–	–	–	–
An. insulaeflorum	–	–	–	–	+	–	–	–	–
An. kochi	+	–	–	+	+	–	–	–	–
An. roperi	–	–	–	+	+	–	–	–	–
An. sundaicus	+	+	–	+	+	+	+	+	–
Ae. aegypti	+	+	+	+	–	+	–	–	–
Ae. albopictus	+	+	+	+	–	+	–	–	–
Ae. albolateralis	+	+	–	+	–	+	–	–	–
Ae. edwardsi	–	+	–	+	–	+	–	–	–
Ae. cancricomes	–	–	–	–	–	–	–	–	+
Ae. simplex	–	–	–	–	–	–	–	–	+
Ae. jamesi	+	+	–	+	–	–	–	–	–
Ae. malayensis	–	+	–	+	–	+	–	–	–
Ae. scutellaris	+	+	–	+	–	–	–	+	–
Armigeres	–	+	–	–	–	+	–	–	–
Culex	+	+	+	+	–	–	–	–	–
Harpagomyia	–	–	–	+	–	–	+	–	–
Hodgesia	–	–	–	–	–	+	–	–	–
Mansonia	+	–	–	–	–	–	–	–	–
Orthopodomyia	–	–	–	–	–	+	–	–	–
Toxorhynchites	–	–	–	–	–	+	–	–	–
Uranotaenia	–	–	–	–	–	+	–	–	–

The immature samples were reared in an insectary until emergence. All adult mosquitoes including those emerged were anaesthesized with ether and identified at Malaria Research Centre, Car Nicobar and Delhi, using the keys of Christophers[3], Barraud[4] and catalogue of Knight and Stone[5]. The mosquitoes were preserved by using 1,4-dichlorobenzene, which is an antifungal agent.

RESULTS AND DISCUSSION

A total of 11358 mosquitoes belonging to 31 species and 10 genera, viz. *Anopheles, Aedes, Armigeres, Culex, Harpagomyia, Hodgesia, Mansonia, Orthopodomyia, Toxorhynchites* and *Uranotaenia* were collected in the survey. The number of specimens and species collected in each genus are given in Table 2. Identification of all the mosquitoes revealed that genus *Anopheles* consisted of six species, *Aedes* nine species, *Armigeres* two spe-

Table 2. List of mosquitoes collected from Car Nicboar Island

Species collected	Total specimens
Anopheles (An.) barbirostris van der Wulp, 1884	1759
An. (An.) barbumbrosus Strickland and Chowdhury, 1927	295
An. (An.) insulaeflorum Swell and Swell, 1919	285
An. (Cellia) kochi Doenitz, 1901	565
An. (An.) roperi Reid, 1950	593
An. (Cellia) sundaicus Rodenwaldt, 1925	6609
Aedes (Stegomyia) aegypti Linnaeus, 1762	96
Ae. (Stegomyia) albopictus Skuse, 1894	69
Ae. (Stegomyia) albolateralis Theobald, 1908	38
Ae. (Stegomyia) edwardsi Barraud, 1923	49
Ae. (Cancraedes) cancraedes Edwards, 1922	59
Ae. (Cancraedes) simplex Theobald, 1903	56
Ae. (Aedimorphus) jamesi Edwards, 1914	49
Ae. (Stegomyia) malayensis Colless, 1962	21
Ae. (Stegomyia) scutellaris Walker, 1959	80
Armigeres (Ar.) kuchingensis Edwards, 1915	89
Ar. (Ar.) subalbatus Coquillett, 1898	40
Culex (Cx.) gelidus Theobald, 1901	48
Cx. (Eumelanomyia) malayi Leicester, 1908	45
Cx. (Culicimoyia) pallidothorax Theobald, 1905	42
Cx. (Cx.) quinquefasciatus Say, 1823	186
Cx. (Cx.) sitiens Wiedemann, 1828	75
Cx. (Cx.) tritaeniorhynchus Giles, 1901	26
Cx. (Lutzia) fuscanus Wiedemann, 1828	35
Harpagomyia genurostris Leicester, 1908	3
Hodgesia malayi Theobald, 1904	2
Mansonia (Mansonioides) uniformis Theobald, 1901	11
Ma. (Mansonioides) indiana Edwards, 1930	8
Orthopodomyia flavithorax Barraud, 1927	68
Toxorhynchites (Toxorhynchites) splendens Wiedemann, 1819	51
Uranotaenia atra Theobald, 1905	6
Total	11,358

contd...

Table 2. ***(contd.)***

Table 3. Per cent composition of different anopheline species in different breeding habitats

Breeding sites	Species emerged					
	An. sundaicus	*An. barbibrostris*	*An. barbumbrosus*	*An. kochi*	*An. roperi*	*An. insulaeflorum*
Creek	2673 (100.00)	0	0	0	0	0
Marshy area	848 (64.30)	386 (29.26)	0	0	0	85 (6.44)
Sunlit stream	530 (24.70)	990 (46.13)	283 (13.19)	0	190 (8.85)	153 (7.13)
Shaded stream in deep jungle	121 (14.07)	251 (29.19)	0	128 (14.88)	313 (36.39)	47 (5.47)
Ponds	440 (92.05)	38 (7.94)	0	0	0	0
Underground cement tanks	249 (93.61)	17 (6.39)	0	0	0	0
Wells	189 (100.00)	0	0	0	0	0
Mangrove	681 (100.00)	0	0	0	0	0
Pits	482 (100.00)	0	0	0	0	0
Jungle pools	21 (6.58)	35 (10.98)	12 (3.76)	251 (78.68)	0	0
Coral reaf cavities	189 (100.00)	0	0	0	0	0
Canoe	228 (100.00)	0	0	0	0	0
Rice field inside the deep jungle	0	0	0	186 (100.00)	0	0
Total	6651	1717	295	565	503	285

Figures in parentheses indicate percentages.

cies, *Culex* seven species, *Harpagomyia* one species, *Hodgesia* one species, *Mansonia* two species, *Orthopodomyia, Toxorhynchites* and *Uranotaenia* one species each.

Among anophelines the most predominant species was *An. sundaicus* (6609 specimens), followed by *An. barbirostris* (1759), *An. roperi* (593) and *An. kochi* (565). Among culicines the major prevalent species was *Culex quinquefasciatus* (186 specimens), followed by *Cx. sitiens* (75) and *Cx. gelidus* (48). *Aedes aegypti* (96 specimens) was the most prevalent species in the genus *Aedes* and it was followed by *Ae. scutellaris* (80) and *Ae. albopictus* (69). In genus *Mansonia* the most common species was *Mansonia uniformis* (11 specimens), followed by *Ma. indiana* (8). The other seven genera, viz. *Armigeres, Ar. kuchingensis* (89 specimens) and *Armigeres, Ar. subalbatus* (40 specimens), genus *Harpagomyia, Ha. genurostris* (3 specimens), genus *Hodgesia, Ho. malayi* (2 specimens), genus *Orthopodomyia, Or. flavithorax* (68 specimens), genus *Toxorhynchites, Tx. splendens* (51 specimens) and genus *Uranotaenia, Ur. atra* (6 specimens) were also encountered.

Basu[1] reported the presence of two anopheline species in Nicobar namely, *An. sundaicus* and *An. philippinensis*. Survey carried out by Krishnan and Bhatnagar[2] also revealed the presence of two anopheline species, viz. *An. sundaicus* and *An. barbirostris*. In the prersent survey a total of six anopheline species were recorded. We recorded four species for the first time in this Island, viz. *An. barbumbrosus, An. insulaeflorum, An. kochi* and *An. roperi*.

Habitat-wise per cent composition of anopheline species found in the samples collected from different breeding habitats have been summarised in the Table 3 and Fig. 3. It was observed that creeks supported the maximum breeding of *An. sundaicus* followed by marshy area, mangroves, ponds, pits, cement tanks, streams, canoes, coral reaf cavities, pools and wells (Table 3). *An. sundaicus* breeds in brackish water and fresh water. But profuse breeding was encountered in the brackish water with free floating and submerged algae. It was observed that putrefying masses of algae/vegetation provide the ideal condition for the breeding and growth of *An. sundaicus*. Our observations revealed that during summer season when biological control agents, viz. *Gambusia affinis* and *Bacillus sphaericus* were released in all aquatic habitats the breeding of *An. sundaicus* shifted to plastic tanks kept over the ground for water storage and in big coconut tree holes. The changes in the breeding habit of *An. sundaicus* is the result of one observation during summer season. In addition to this the breeding of *An. sundaicus* was observed in canoe, coral cavities/holes. This is the first report of *An. sundaicus* breeding in coral cavities.

The breeding of *An. barbirostris* was observed in streams, marshy area, ponds, jungle pools and cement tanks. Breeding of this species was associated with vegetation, decaying leaves or organic matter. *An. barbiro-*

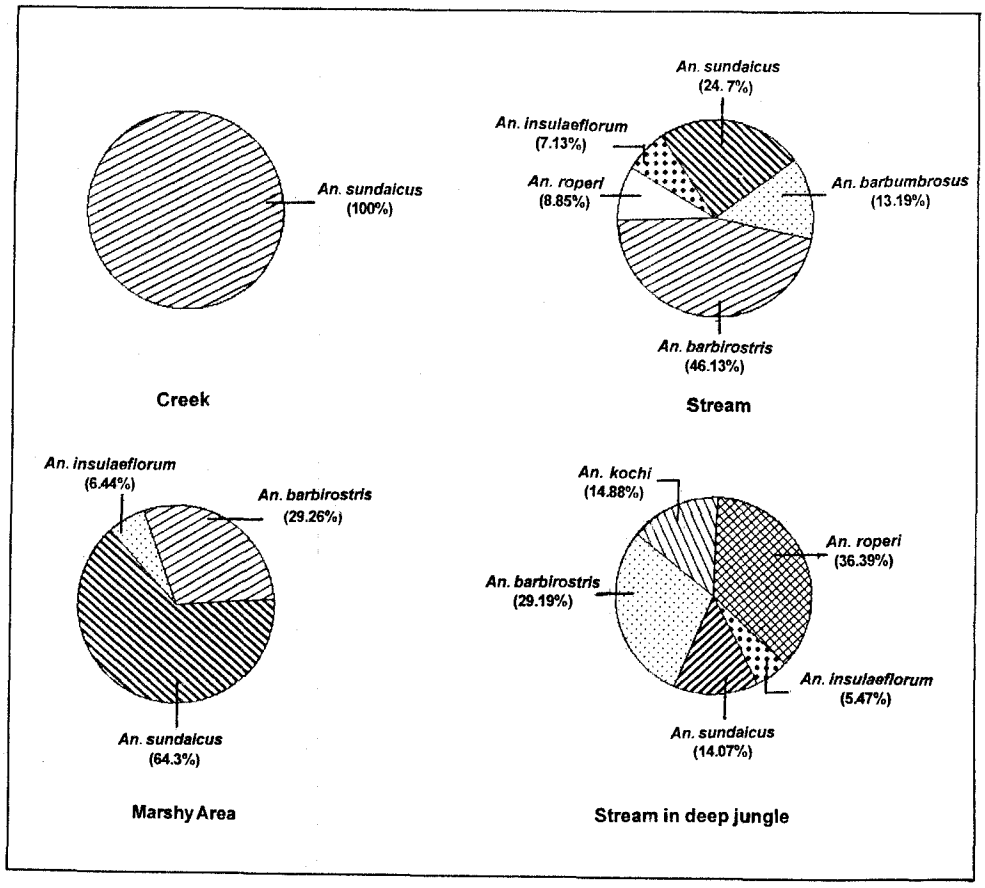

Fig. 3: Habitat-wise composition of anophelines

stris was the second most predominant species.

Stream margins and jungle pools supported the breeding of *An. barbumbrosus*. Streams, pools, rice fallows, water patches inside the deep jungle supported the breeding of *An. kochi*. Deep jungle shaded stream, margin of flowing stream, shaded pools near the stream were breeding sites of *An. roperi*. Shaded streams, marshy area inside the jungle, plant roots associated with decaying leaves in the streams were the breeding places of *An. insulaeflorum*.

In addition to the mosquito fauna survey, the present study highlights the breeding status of different *Anopheles* species with reference to *An. sundaicus* which is the malaria vector in Car Nicobar Island. Based on this study anti-larval operation, such as the alternative approach to non-insecticide strategy, viz. bioenvironmental control of malaria is being implemented in the Island.

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REVIEW ARTICLE

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Microbial Control of Mosquitoes with Special Emphasis on Bacterial Control

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There are a number of microbial agents including fungus, protozoa, virus and bacteria which act as mosquitocidal agents. However, among these agents, *Bacillus thuringiensis* var *israelensis* and *B. sphaericus* are the most potent mosquitocidal agents. *Bacillus thuringiensis* var *israelensis* and *B. sphaericus* are gram-positive sporulating bacteria which produce protoxin crystals during sporulation and are highly toxic to susceptible mosquito larvae when they ingest them. These bacterial agents are environmentally safe due to their host specificity, require in very low dosage, easy to prepare commercially in large-scale and are less costly. Field trials with various formulations of *B. sphaericus* and *B. thuringiensis* var *israelensis* have demonstrated their safety and potential for controlling mosquitoes. Moreover, cloning and expression of the toxin genes of these organisms in other environment friendly bacteria have also made them important for further investigation.

Recent advances with novel types of recombinant micro-organisms with new cloning strategies and cloning the toxin genes under strong promoter for over expression together with *in vitro* gene manipulation and site directed mutagenesis of the active sites for increased toxicity have the potential to provide more effective control of mosquitoes by exploiting these two bacteria. But the toxins of *B. sphaericus* and *B. thuringiensis* var *israelensis* in particular, do not persist long in nature and require frequent application which is a limiting factor for these organisms to be most successful and potent biolarvicide. Nevertheless, they are by far the best choice for controlling mosquitoes. In this review article different mosquitocidal agents with particular emphasis on *B. thuringiensis* var *israelensis* and *B. sphaericus* have been described.

Keywords: *Bacillus sphaericus*, *Bacillus thuringiensis* var *israelensis*, Bacteria, Cloning and expression, Formulations, Fungus, Mode of action, Mosquito, Protozoa

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INTRODUCTION

Some of the world's most dreaded diseases are transmitted by mosquitoes. Members of the sub-families Anophelinae and Culicidae are the most important disease vector. Malaria, filaria parasites and a few arboviruses are transmitted by *Anopheles* mosquitoess[1]. Among the Culicidae sub-family, genus *Culex, Aedes, Mansonia* and *Armigeres* are vectors of Filariasis, Japanese encephalitis, Yellow fever and Dengue[1]. Malaria, the most common and debilitating disease is transmitted by female Anophelinae mosquitoes.

Chemical insecticides such as gammaxane, dichlorodiphenyl trichloroethane (DDT), malathion, chlorodane etc., are conveniently used to control mosquitoes. Antimalarial drugs, e.g. quinine, chloroquine, pyrimethamine, primaquine, combination of amodiaquine-sulphadoxine are used to control malaria. But the emergence of insecticide resistant vectors and drug-resistant parasite has led to the resurgence of malaria particularly in tropical countries with increasing heavy annual death toll. In addition, the emergence of pesticide and drug-resistant mosquitoes, coupled with long-term detrimental effects of powerful chemicals to non-pest insects and concern about accumulation of pesticides in the food chain and environment, has prompted for extensive search for alternative control and an effective and promising alternative is biological control.

In nature, a wide variety of organisms including viruses, protozoa, fungi and bacteria are effective against mosquitoes[2]. But the viral, protozoan and fungal agents were not commercially successful as mosquitocidal agents due to the labour and costs associated with their *in vitro* large-scale production.

The bacterial strains which produce mosquitocidal toxins are *B. sphaericus* and *B. thuringiensis* var *israelensis* and have emerged to be most potent biolarvicidal agents against mosquitoes with no detrimental effect on non-target organisms due to their host specificity and environmentally and ecologically safe. The molecular potency of the bacterial larvicides is high compared to that of chemical insecticides and the toxin degrades in a few days after their action is complete and the bacteria appear to be safe for other insects, animals and the environment. Moreover, the bacterial larvicides are cost-effective and easy to produce commercially in large-scale.

In this review article emphasis will be given mainly though not exclusively on bacterial control of mosquitoes.

Viruses

Members of the Baculoviridae (nuclear polyhedrosis virus), Reoviridae (cytoplasmic polyhedrosis viruses), Poxiviridae, Parovoviridae and Iridoviridae are effective against mosquitoes[3,4]. However, their uses were not that much successful and none has been economically produced in large quantities to control mosquitoes.

Protozoa

Numerous protozoa are active against mosquitoes in nature and the most common and best studied are the microsporidia (Microspora)[5-7]. Species such as *Hazzardia milleri, Culicospora magna, Culicosporella lunata* have dimorphic spores and represent dimorphic microsporidia[8,9]. However, these protozoa are unlikely to be developed as effective mosquitocidal agents owing to labour and expense associated with their *in vivo* large-scale production.

Fungi

Three genera of fungi, Lagenidium, Coelomomyces and Culicinomyces were found to be effective against mosquitoes.

Lagenidium giganteum: *Lagenidium giganteum* is a pathogen of mosquitoes. Zoospores are the infective stage of the fungus[10]. Improvements in artificial media have resulted in efficient *in vitro* production of zoospores. Several factors, e.g. temperature, organic pollution, salinity, pH, anaerobic conditions, the presence of other microbes and also age, density and species of the target population influence the level of pathogenicity[11-13].

Coelomomyces: *Coelomomyces* (Blastocladiales) are potential control agents against mosquitoes because of their broad host range. A major hurdle to large-scale use of *Coelomomyces* for mosquito control is dependent on *in vivo* production as the only source of inocula. Inoculation of mosquito habitats with sporangia resulted in highly variable infections[14]. High level of mortality in mosquito larvae over a broad geographic areas were reported after inoculation of habitats with infective material[15].

Culicinomyces clavisporus: *Culicinomyces clavisporus* (Hyphomycetes) has very broad mosquito host range[16-18] and are very suitable for culture and mass production of the three most promising candidate for controlling mosquitoes[16,19-21]. Anophelines are less susceptible than culicines and *Aedes aegypti* is more susceptible than *Culex quinquefasciatus*[16,21,22]. An obvious drawback of *C. clavisporus* is the high dosage required for effective control[23].

Bacteria

Certain bacteria, particularly member of the genus *Bacillus,* e.g. *Bacillus thuringiensis* var *israelensis* and *B. sphaericus* have been successfully used for controlling mosquito population.

Bacillus sphaericus: *B. sphaericus* is commonly found in a variety of soil and aquatic environments[24-27]. During sporulation, some strains of *B. sphaericus* produces parasporal inclusion or "crystal" which contains proteins toxic for larvae of a variety of mosquito species[28-29]. The major components of the crystal are two proteins of 51 and 42 kDa[30] and both are required for toxicity[31-33]. Toxicity levels vary among the larvicidal serotypes and even within the same serotype[34]. So far a number of strains of different serotypes have been isolated with

mosquitocidal activity[25,29,35]. The most widely studied strains belong to serotype H-2 (e.g. strain SS II-1), serotype H-6 (e.g. strain I AB 59), serotype H-25 (e.g. strain 2297), serotypes H5a, 5b (e.g. strains 1593 and 2362).

Location of the crystal genes

Several studies indicate that toxin genes of *B. sphaericus* are located on bacterial chromosomes[36-40]. Strain 1691 lacks detectable plasmids indicating that the toxin genes are located on the chromosome[40].

Toxins

Mosquito larvicidal activity of highly toxic *B. sphaericus* is due to the presence of approximately equal amount of 42 and 51 kDa proteins[41] both of which are required for toxicity[28,30,32,42]. However, purified forms of these two protein exhibit much lower toxicity[43]. It therefore appears that spore components or other as yet unidentified components may play a role in toxic activity. Compared to highly toxic strains of *B. sphaericus*, such as 2362, 2297 and 1593, strains SS II-1 has much lower toxic activity and the toxins are unstable[44].

Cloning and expression of toxin genes

The genes coding for 42- and 51-kDa protein of *B. sphaericus* have been cloned and expressed[31,36,45-49]. Both the genes were found within a 3.0 to 3.5 kb Hind III fragment[39]. Enhanced expression of mosquito larvicidal toxin genes of 1593 M strain of *B. sphaericus* have been observed in *E. coli*[50]. Toxin genes from *B. sphaericus* 1593 was also cloned and expressed in *B. subtilis*[51].

Mechanism of toxin action

Experimental evidences strongly suggests that both 51- and 42-kDa proteins of *B. sphaericus* at optimum proportion of 1:1 are required for toxic activity[43,52,53]. The toxins bind in approximately equal amounts to the gastric cecum and posterior midgut[53] and involve the following series of action: (i) Ingestion of the crystal protoxins by the larva; (ii) Solubilization of the crystal by the alkaline pH in the midgut; (iii) Proteolytic cleavage of the 42- and 51-kDa protoxins to ca 39- and 43-kDa active components; (iv) Binding of the active toxin to epithelial cells of gastric cecum (GC) and posterior midgut (PMG); (v) Internalization of both toxins followed by appearance of areas of low electron density, vacuolation and mitochondrial swelling; and (vi) Lysis of cells[54,55].

Binding studies with purified 42- and 51-kDa protein revealed that there was localized binding to the PMG and GC when the 51-kDa protein was fed alone to *Culex* larvae, whereas the 42-kDa protein alone showed non-specific binding throughout the gut and GC. In the presence of 51-kDa protein, 42-kDa protein localized to the PMG and GC, i.e. where the 51-kDa alone bound. It has, therefore, been proposed that 51-kDa protein acts as the cell-binding component and directs and binding of the 42-kDa pro-

tein. Since 51- and 42-kDa proteins associate very strongly with each other[53], it is plausible that both 42- and 51-kDa proteins bind simultaneously to the receptor sites and cell-membrane which results in a suitable configurational change necessary for internalization of both proteins. It is believed that 51-kDa protein may have a dual role in cell binding and toxic action[53,55-57]. The binding of both proteins to specific receptor site has been demonstrated by the fact that none of the protein binds to PMG and GC of the toxin-resistant mosquitoes and to the gut of non-susceptible *Ae. aegypti* larvae[55-57].

The presence of the N-terminus of the 51-kDa protein between amino acids 39 and 45 is required for its localized binding to the PMG and GC[55]. Deletion studies of the 51-kDa protein showed that N-terminal region is necessary for receptor binding and the C-terminal region is required for binding of the 42-kDa protein and interaction of both the protein is necessary for toxicity[55]. Experimental evidences do suggest that the 42-kDa protein is responsible for the host range whereas overall toxicity is the function of both proteins[27,36,58].

The 100-kDa mosquitocidal toxin of *B. sphaericus* SS II-1 does not exhibit homology with any known insecticidal toxin but shows homology with pertusis toxin and diphtheria toxin[59].

The pertusis toxin and diphtheria toxin exert their action by ADP-ribosylation of cellular proteins and the homologies among 100-kDa and these toxins do suggests that 100-kDa toxin likewise may act by ADP-ribosylation[60].

Expression of toxins in other bacteria

B. sphaericus toxin genes were expressed in *Escherichia coli* and other *Bacillus* species. Recombinant binary toxins were expressed in *E. coli* JM105, *B. subtilis* DB104, non-toxic *B. sphaericus* 713. *B. sphaericus* SS II-1 toxin genes were expressed in both crystalliferrous and a crystalliferrous *B. thuringiensis* subsp *israelensis*[31,42-45,53,55,61]. In some cases high level expression was observed. Recently, a truncated 100-kDa toxin lacking the non-essential N-terminal putative signal peptide was expressed in *E. coli* and was found to be equally toxic to 42- and 51-kDa protein combination as determined by LC_{50} value. The genes encoding *B. sphaericus* binary toxin were also expressed in heterologous bacteria such as *Caulobacter crescents* CB-15[62], cyanobacteria[47], *Asticcacaulis excentricus* and the recombinant bacteria exhibited toxicity against *Culex* and *Anopheles* mosquito larvae[63].

Resistance against *B. sphaericus* toxins

Laboratory selection of *Cx. quinquefasciatus* against *B. sphaericus* has been achieved. Direct binding experiments with isolated brush-border membrane fractions from larvae of a susceptible laboratory strain of *Cx. quinquefasciatus* indicated the presence of a single class of toxin receptors. *Cx. quinquefasciatus* resistant to *B. sphaericus* bi-

nary toxin failed to reveal the presence of any specific binding. The resistant strain had lost the functional receptor for toxin. The binding characteristic of brush-border membrane fractions from the F_1 larval progeny (susceptible females x- resistant males) were very close to those of parental susceptible strain, consistent with resistance being recessive[64]. Field trial of *B. sphaericus* revealed that, it was very effective initially, but within a year, after 20-25 rounds of application, field population of *Cx. quinquefasciatus* developed resistance up to 150-fold. Genetic studies revealed that resistance was recessive, autosomal and controlled by more than one gene[65]. *Cx. quinquefasciatus* resistant to *B. sphaericus* was also observed by other investigators[66,67].

Formulations

Several formulations of *B. sphaericus* for successful field application were developed, e.g. water-dispensable powder (WDP), flowable concentrate (FC), granules and dust. In most of the cases the larval control was restricted to 24-48 h only. Use of formulations such as briquettes, polypropylene pellets, alginate beads and microgels resulted in appreciable level of control for 2 to 8 wk. The mosquito species tested with these formulations include, *An. gambiae, An. subpictus, Cx. stimatosoma, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Mansonia* spp and *Psorophora columbiae*. *B. sphaericus* has a prolonged larvicidal activity due to recycling and amplification of spores in larval cadavars and certain aquatic situations or may be due to the long-term persistence of sufficient and accessible toxin in the environment; or it may be due to combination of the above[68-70]. Field application and laboratory study of powder formulation of *B. sphaericus* strain B-101, serotype H-5a 5b against *An. stephensi* and *Cx. quinquefasciatus* showed 100% mortality in laboratory condition at room temperature and persisted over 12 wk for both the mosquitoes[71]. Field evaluation exhibited 95-100% reduction in larval density of both the mosquitoes within 48 h in different habitats, and the larvicidal activity persisted for two to four weeks in water habitats[72]. Temperature and pH greatly affects the larvicidal activity of *B. sphaericus* formulations[73,74].

Bacillus thuringiensis var *israelensis*

B. thuringiensis var *israelensis* produces delta-endotoxin which are toxic against mosquito and blackfly larvae[75,76]. Other than *B. thuringiensis* var *israelensis* serotype H-14, many novel subsp have recently been isolated and characterized. Ragni *et al.*[77], have isolated six highly mosquitocidal *B. thuringiensis* strains that do not belong to serotype H-14. Another novel strain was isolated and characterized[78]. Phage typing of serovars of *B. thuringiensis* var *israelensis* also revealed many new strains[79].

Crystal formation and sporulation

Several studies revealed that the expression of the crystal protein gene is functionally related to sporulation specific events[80].

However, sporulation and crystal formation could be delinked[81] and recent studies[82,83] showed that sporulation and crystal formation are not inextricably linked and crystal protein (delta-endotoxin) gene(s) expression is independent of sporulation specific functions. Subsequent studies[84,85] supported this.

Bacillus thuringiensis var *israelensis* toxins

The crystal toxins of *B. thuringiensis* var *israelensis* are composed of atleast five polypeptides of approximately 134, 128, 70, 58 (a minor component), and 27 kDa[86,87]. Chilcott and Ellar[86] purified crystal proteins to yield 130, 65, 28, 53, 30-35 and 25 kDa proteins (130, 65 and 28 kDa proteins and their proteolytic products 53, 30-35 and 25 kDa proteins) and found that all the purified proteins were toxic against *Ae. aegypti* larvae. None of the crystal proteins of *B. thuringiensis* var *israelensis* is as toxic as the intact crystal complex containing all the proteins[87,88]. The 134, 128, 58 and 70 kDa proteins are all toxic to *Ae. aegypti* larvae, and the 70 kDa protein has moderate activity against *Cx. pipiens*[89-94].

Greater toxicity of the intact crystal protein complex compared to individual components suggests that synergism may be an important factor for toxicity and has been demonstrated by Crickmore *et al*[95,96].

Toxin genes of *Bacillus thuringiensis* var *israelensis*

Several genes coding crystal proteins have been identified. The 134, 128, 70, 58 kDa proteins are coded by *cry IV A, cry IV B, cry IV D* and *cry IV C* genes respectively[54]. The 27 kDa protein with hemolytic activity is coded by *cyt A* gene. The *cry IV A* and *cry IV B* genes each encode different 130 kDa proteins (designated 134 and 128 kDa proteins respectively) which are proteolytically cleaved into toxic N-terminal core fragments of 53 to 67 kDa. The *cry IV C* gene encodes an initial translation product of 77.8 kDa and bears some homology to the 5' half of the *cry IV A* and *cry IV B* genes[92]. The *cry IV D* gene encodes a 72.4 kDa protein which is a major component of the toxic crystal. The 72.4 kDa protein is proteolytically activated to ca 30 kDa protein.

Location of the crystal genes

The gene(s) coding for crystal protein in different species and subspecies of *B. thuringiensis* are plasmid encoded[97] although examples of chromosomal location of the gene(s) have also been reported[98]. The number of plasmids in different strains of *B. thuringiensis* varies from 2 to 12 with sizes ranging from ca 1.5 to 150 MDa[100]. The crystal protein genes are mostly located in large plasmids (15-120 MDa)[99]. The crystal protein coding genes (*cry IV* class) and the gene coding for 27-kDa cytolytic protein (*cyt A* class) in *B. thuringiensis* var *israelensis*, are located on the same 72-MDa plasmid[100].

Cloning and expression of toxin genes

Crystal protein gene(s) of *B. thuringiensis*

var *israelensis* have been cloned and expressed in *E. coli* and the extracts of *E. coli* cells harbouring the recombinant crystal protein genes found to be toxic to mosquito larvae[101-104]. Expression of plasmid borne delta-endotoxin genes of *B. thuringiensis* var *israelensis* were also observed in both wild type sporogenic and mutant asporogenic strains blocked at very early stage of sporulation of *B. subtilis*[105]. Plasmids coding crystal protein genes were also transferred to sporogenic and asporogenic mutant strains of *B. cereus* and the transconjugants were found to be toxic to *Ae. aegypti* larvae[106].

The 134-kDa (*cry IV A*), 128-kDa (*cry IV B*), ~58-kDa (*cry IV C*) and 70-kDa (*cry IV D*) protein coding genes were individually cloned and expressed in heterologous bacteria and the recombinant proteins were found to be toxic to *Ae. aegypti* larvae to various degrees and 70-kDa protein was found to have moderate toxicity against *Cx. pipiens*[89-93,107]. *B. thuringiensis* var *israelensis* crystal protein genes were also expressed in *B. subtilis* and non-toxic mutant strain of *B. thuringiensis* var *israelensis*[92,108]. The *cry IV D* crystal protein gene of *B. thuringiensis* var *israelensis* has been cloned and expressed in *B. megaterium*[90].

Increased larvicidal activity of *E. coli* with combinations of genes from *B. thuringiensis* subsp *israelensis* were also observed[109]. *Bacillus thuringiensis* var *israelensis* toxin genes were also expressed in *B. sphaericus* to increase the insecticidal activity[93,110,111]. The 27-kDa (*cyt A*) and 70-kDa (*cry IV D*) toxins have been cloned and expressed in both *B. subtilis* and the high-toxicity strain *B. sphaericus* 2362[110]. The 128-kDa *cry IV B* toxin from *B. thuringiensis* var *israelensis* was also expressed in *Caulobacter crescentus* CB-15[62]. Toxin genes were also expressed in cyanobacteria but the toxicity against susceptible hosts were found to be very low[47,112,113]. Expression of 128-kDa *cry IV B* protein was also achieved in *Synechococcus* sp strain PCC 6803[113]. *B. thuringiensis* var *israelensis* toxin gene was also expressed in Baculovirus vector[114]. The genetic stability of a cloned toxin gene in a recombinant cell is of much concern for the recombinant cells to be effective so far potency and persistence of toxicity is concerned. Recombinant plasmids are not very stable mostly due to following reasons: (i) Loss of plasmid populations during segregation; (ii) Structural instability due to plasmid re-arrangements and deletions; and (iii) Plasmid incompatibility.

In most cases toxicity of the recombinant cells were found to decline after few generations. Therefore, attempts have been made to isolate hyper-toxic mutant strains by *in vitro* mutagenizing the parent strain with mutagens. One such hyper-toxic mutant strain of *B. thuringiensis* var *israelensis* were found to be stable for generations[115].

Mechanism of action of toxins

The mode of toxin action has been studied both on brush-border midgut membranes[116]

and on cultured insect cells[117]. These studies, together with studies with susceptible larvae, have revealed the following series of steps: (i) Ingestion of crystal-spore complex; (ii) Solubilization of the crystal in the midgut by the alkaline pH; (iii) Clevage of the protoxins into active toxic components called delta-endotoxin by gut proteases; (iv) High-affinity binding of the activated toxin to specific brush-border membranes; (v) Induction of non-specific pores, which function as leakage channels, resulting in the net influx of ions and inflow of water; and (vi) Cell swelling and colloid osmotic lysis resulting in larval death.

The toxin's mode of action can be attributed, in part, to its three dimensional structure.

B. thuringiensis var *israelensis* parasporal inclusions have structural disulfide bonds and the *cry IV A* and *cry IV B* proteins require disulfide reduction for solubilization[118]. The 27-kDa *cyt A* protein aggregates as a trimer or tetramer, apparently because of disulfide bonds[118].

The larger protoxins of approximately 130-140 kDa, required processing to active toxins[88] mediated by the alkaline pH and proteases of the insect midgut. This action yields 60-70 kDa proteinase-resistant toxin fragments derived from the N-terminal half of protoxin[119-121]. The degradation occurs in an ordered sequence with cleavages starting at the C-terminal end proceeding towards the N-terminal region. The C-terminal fragment of the protoxin probably required for the formation of parasporal inclusion. The activated toxin can be divided into three structural regions: (i) a N-terminal region, the toxic domain (amino acids 1-279), consisting of several conserved region which are hydrophobic; (ii) a conserved C-terminal region (amino acids 461-695); and (iii) a variable region between these two regions. The N-terminal domain plays a significant role in toxicity. The C-terminal domain may be needed for toxin to bind specifically to cell receptors[122].

The toxin binds specifically to brush-border membrane vesicles (BBMV) which consists primarily of the apical brush-border membrane of the midgut columnar cells. Insect cells probably have multiple toxin receptors and toxicity is most probably related to receptor number than receptor affinity[123,124]. It is believed that the receptor is a glycosylated protein[117,125,126]. The *cyt* toxin has a different cell-membrane interaction from that of the *cry* toxins[127-130]. Initial binding of *cyt* toxin occurs with unsaturated phospholipids[128,130]. Initially, the toxin binds as monomer, upon continued exposure, aggregates of the toxin form in the cell-membrane[127,131].

Activated toxins apparently insert in the midgut membrane and increase the K^+ conductance of the columnar cell apical membrane which leads to disruption of electrical, K^+ and pH gradients[116,132].

It has been proposed that the activated toxins, first directly inhibit plasma membrane ATPases[133] which regulates K^+ transport and

potential gradient across the membrane[134]; second, the toxins increase K^+ permeability selectively[132] or the toxins form a non-specific pores[135,136]. However, it is more likely, that the toxins form non-specific pores.

Three factors govern the host specificity. First, difference in the larval gut which affect the solubility of the crystal complex; second, the efficiency with which the protoxin is processed and third, the expression or availability of putative membrane receptors for the toxins in the gut of the insect.

Comparison of amino acid sequences of the N-terminal half of the *cry IV* toxins revealed that the sequences of the *cry IV A*, *cry IV B* and *cry IV C* toxins share significant homology which is restricted largely to distinct regions named Block 1 to 5[88] and the core of the mosquitocidal toxin is built of these conserved sequences.

Mutational studies[137-138] led to the prediction that domain-I is required for membrane insertion and the inhibition of certain ion-dependent transport processes. Site directed mutagenesis studies led to the proposal that domain-II determines host specificity[139]. The specificity – determining regions of various *cry I* toxins found to map largely within domain-II[140,141]. There are five amino acid sequences highly conserved in *B. thuringiensis* delta-endotoxins. Mutational changes of amino acid residues in Block 5 revealed that Block 5 of *cry IV A* is one of the stability-determining elements of the protoxin molecule[142]. The protoxins are composed of two domains, an amino-terminal half essential for toxicity and a carboxy-terminal half with an as yet unassigned function. Mutational analysis revealed that the non-toxic domain is required to direct the synthesis of inclusion bodies as an active soluble form and solubilization under alkaline conditions in the insect midgut is considered to be the first step for toxic action[143]. Mutations in the third domain of *B. thuringiensis* delta-endotoxin *cry IA A* was found to affect its ability to increase the permeability of the midgut brush-border membrane vesicles[144].

Resistance against toxins

Significant resistance against *B. thuringiensis* var *israelensis* has not been observed during its use over more than two decades[145]. However, laboratory selection of some mosquitoes of *Ae. aegypti* and *Cx. quinquefasciatus* has been achieved though not easily. Gill *et al.*[122] observed high level of resistance of *Cx. quinquefasciatus* against *cry IV D* toxin of *B. thuringiensis* var *israelensis*. Receptor binding studies with laboratory-selected resistant *Paodia interpunctella* strain to *B. thuringiensis* insecticidal crystal protein is correlated with a 50-fold reduction in affinity of the brush-border membrane receptor for this protein[124]. It seems that modification of the binding sites is a major mechanism of resistance to *B. thuringiensis*[124,146].

Formulations

Different formulations of *B. thuringiensis* var *israelensis* have been used, e.g. water

dispensable powder (WDP), flowable concentrate (FC), granules and dust. These formulations have been used to control anophelines[147], although the residual activity of these formulations was found to be limited to 24-48 h post-treatment. Certain slow release formulations such as briquettes, polypropylene pellets, alginate beads and microgels have been found to provide significant level of control with residual activity extending to 2 to 8 wk. Previously it was believed that *B. thuringiensis* var *israelensis* does not persist long in nature and are unable to recycle and needs frequent application which is a limiting factor for this toxin. The efficacy of a potential toxin depends on long persistence, ability to recycle and long-lasting effects on insects. However, recent study has shown that *B. thuringiensis* var *israelensis* toxin are able to recycle in larval gut.

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LETTER TO THE EDITOR

Indian Journal of Malariology
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Prevalence of Malaria and ABO Blood Groups in a Seaport Area in Raigad, Maharashtra

Sir — During 1995 and 1996 there was resurgence of malaria in different parts of the Raigad district located in the coastal region of Maharashtra. As a part of the investigation, we conducted a malaria survey around the Jawaharlal Nehru Port Trust (JNPT) and also studied the ABO system to find out any possible relationship with malaria during May 1996. From previous studies, it is known that human blood polymorphic systems are important biochemical markers in the disease distribution including malaria. In Africa, Luzzatto *et al.*[1] reported the relationship of genetic variants of human red blood cells with malaria. In India, among all systems of blood groups, the ABO system has been studied in various groups in different parts of the country[2-5].

The present report on the prevalence of malaria and distribution of ABO polymorphic system in the JNPT area, the Jawaharlal Nehru Port Trust is a seaport in Raigad which is surrounded by labour colonies (pop. 3500). In this population a rapid fever survey was conducted. About 0.3 ml whole blood of the persons with active fever was taken through aseptic finger-prick in heparinized vials, stored under ice-cool condition in the field and brought to the laboratory for analysis. Thick and thin blood smears were also prepared simultaneously for diagnosis of malaria. ABO blood grouping was done by agglutination slide test. All the febrile patients were given presumptive treatment (600 mg chloroquine base as adult dose) and those found with malaria were given further treatment through the District Malaria Officer, Raigad.

A total of 121 blood samples were taken from the patients of both the sex between the age of 2 and 60 yr. These subjects comprised of caste hindus (scheduled castes, other backward castes and a small group of general castes) and muslims. The frequencies of ABO blood groups among study subjects are given in Table 1. Among the study subjects 24% had blood group 'A', 35.5% group 'B', 17.4% group 'AB' and 23.1% blood group 'O'. These frequencies of ABO system are within the range for Indian population[6]. Among the scheduled castes, blood group 'A' was widely prevalent (31.3%), followed by 'O' and 'B' (25% each) and 'AB' (18.7%). *Deshastha Rigvedi,* Brahmins of Maharashtra state, also showed higher frequency of A and B gene[5]. In other castes blood group 'B' was dominant (36.4%) followed by 'A' (27.2%), 'O' (21.2%) and 'AB' (15.1%). In other backward castes frequency of blood group 'B' was highest (37.5%) followed by 'O' (23.6%), 'A' (28.8%) and 'AB' (18.1%).

Out of 121 subjects included in the study, 34 had malaria (*Plasmodium vivax* 11; *P. falciparum* 21 and their mixed infections 2). The sex-wise distribution showed that malaria prevalence was higher in males (29.9%) than the females (23.5%), however, this difference was statistically non-significant ($\chi 2 = 0.748$; $p > 0.05$). Data on the distribution of malaria positive cases according to their blood groups are given in Table 2. Malaria prevalence was highest in the people with blood group 'B' (34.8%) and lowest in blood group'AB' (19%). Highest *P. falciparum* rate was found in blood group 'O' (6/28; 21.4%) and lowest in 'AB' blood group (2/21; 9.5%). Chi-square test did not show any association of malaria with ABO polymorphism ($p > 0.05$). In earlier studies no association was found between ABO system and malaria in Indian and Colombian populations[2,7]. However, Joshi *et al.*[8] reported that blood group 'AB' was less susceptible to malaria. Pant *et al.*[3] observed that blood group 'B' and 'O' had an advantage against *P. falciparum* malaria.

Table 1. Distribution of ABO blood groups among various population groups

Groups	ABO system				Total
	A	B	AB	O	
Scheduled castes	5 (31.2)	4 (25.0)	3 (18.7)	4 (25.0)	16
Other backward castes	15 (20.8)	27 (37.5)	13 (18.1)	7 (23.6)	72
Other castes	9 (27.2)	12 (36.4)	5 (15.1)	7 (21.2)	33
Total	29 (24.0)	43 (35.5)	21 (17.4)	28 (23.1)	121

Figures in parentheses are percentages.

Table 2. Prevalence of malaria by blood groups

ABO system	Healthy		Malaria cases			Total
	Total	Individuals	*Pv*	*Pf*	Mixed	
A	29	21 (72.4)	3	5	0	8 (27.6)
B	43	28 (65.1)	5	8	2	15 (34.8)
AB	21	17 (81.0)	2	2	0	4 (19.0)
O	28	21 (75.0)	1	6	0	7 (25.0)
Total	121	87 (71.9)	11(9.1)	21(17.3)	2 (1.6)	34 (28.1)
χ^2, df = 3			3.015 NS	1.983 NS		1.968 NS

Figures in parentheses are percentages; NS — Not significant, $p > 0.05$.

To conclude, malaria infection due to *P. vivax* and *P. falciparum* were found in people of all blood groups and none of them had any protective advantage over malaria infection.

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H.C. SRIVASTAVA
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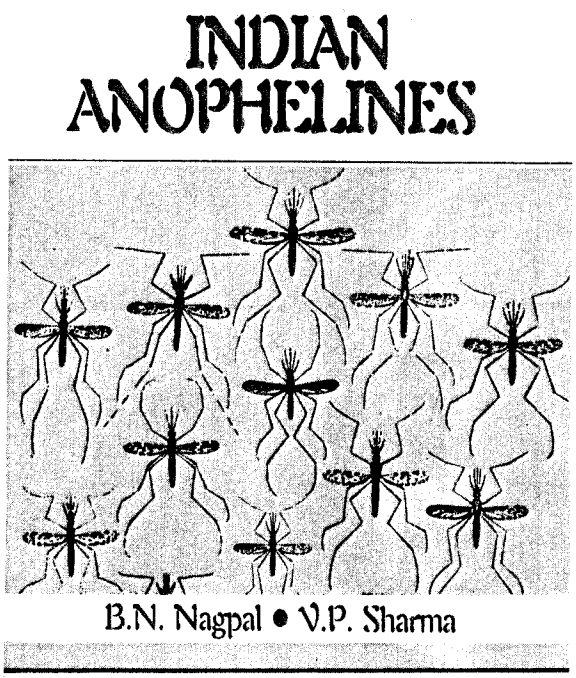

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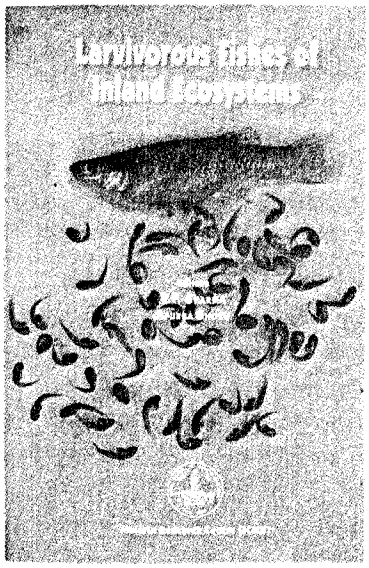

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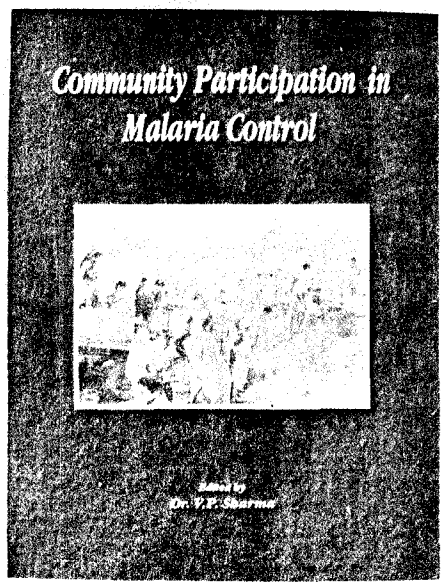

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